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Stimulation of Erythropoiesis in Sublethally Irradiated Rats by a Plasma Factor.* (22152)

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It is well established that red cell production is severely depressed by low doses of whole body irradiation. Erythropoiesis can be significantly increased, however, when the animal is bled immediately before(1,2) or after(2) sublethal irradiation. A similar but less pronounced effect results from the post-irradiation administration of para-aminopropiophenone (PAPP), which produces a severe, transient methemoglobinemia. Recently evidence has been presented to indicate that in the normal animal hypoxia elicits the release of a humoral substance (hemopoietine) capable of stimulating erythropoiesis(3-6). Since both bleeding and PAPP produce hypoxia, it might be suggested that the subsequent release of hemopoietine is responsible for the accelerated regeneration of erythropoietic tissue in the sublethally irradiated animal. If such is the case, plasma collected from donors with post-hemorrhagic anemia might be capable of stimulating erythropoiesis in the sublethally irradiated animal.

Material and methods. Female Sprague-Dawley rats weighing 135-170 g were used. In each experiment 24-30 animals were randomly assigned to 3 or 4 groups. Animals were exposed to whole body irradiation, 200 r from a Co^{60} source in 4 π geometry(7) or 160-190 r from a 2.5 MEV Van de Graaff generator at an average dose rate of 50 r per minute measured with a Bendix ionization chamber known to be air equivalent at that energy. "Anemic" plasma was prepared by bleeding normal donor rats 2% of the body

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* We wish to acknowledge the help of Dr. Howard L. Andrews, National Institutes of Health, and Commander Jasper E. Morgan, Naval Medical Research Institute, who generously provided the facilities for irradiation.

TABLE I. Effect of Plasma from Anemic Donors on Fe^{59} Incorporation in Sublethally Irradiated Rat.

Exp. #	Irradiated controls	Normal plasma	Anemic plasma* 24 hr	Anemic plasma* 48 hr	p†	Radiation Source	Radiation Dose, r
1	23 ± 2.6 (7)‡	36 ± 7.8 (7)		68 ± 2.5 (5)	$<.01$	Co^{60}	200
2	35 ± 1.7 (8)	32 ± 4.4 (7)		42 ± 2.5 (7)	$<.05$	2.5 MEV	160
3	31 ± 6.2 (5)	33 ± 6 (6)	51 ± 1.3 (5)	38 ± 2.9 (6)	$<.01$	2.5 "	160
4		39 ± 6.6 (6)	44 ± 2.7 (8)		n.s.	2.5 "	180
5		38 ± 5 (7)	53 ± 2.1 (8)		$<.01$	2.5 "	180
6	26 ± 4 (5)	37 ± 2.6 (6)	53 ± 4 (7)		$<.01$	2.5 "	190

* Plasma collected 24 or 48 hr after production of post-hemorrhagic anemia.

† p refers to probability that observed differences between groups receiving anemic and normal plasma occurred by chance. In the third experiment, 24 hr anemic plasma was compared with normal plasma. The p was determined using the one sided t test.

‡ Fe^{59} values given represent % of total injected dose present in circulating red cells 48 hr after injection ± a single stand. error computed as $\frac{\text{Range}}{K_n\sqrt{n}}$(10). Number of animals in each group is included in parentheses.

weight (approximately 30% of the blood volume) by cardiac puncture. 24 or 48 hours thereafter, blood was collected in heparin or 5% Na_2 ethylene diamine tetraacetate and the plasma separated. Control plasma was obtained from normal donors not previously bled. The plasma preparation to be tested was injected into the tail vein within 90 minutes following irradiation. An amount equivalent to 30% of the recipient's blood volume, calculated as 7% of the body weight, was injected. For studies of heat stability, anemic plasma was adjusted to pH 5.4 with 1 N HCl and heated at 100°C for 10 minutes. The precipitated protein was separated by centrifugation at 2000 G for 2 hours. The pH of the supernatant was adjusted to 7.1 with 1 N NaOH and injected as before. Fresh anemic plasma was also dialyzed at 4° for 24 hrs. 25 cc of plasma was dialyzed against 1000 cc of saline with 3 changes of the dialyzing fluid during the 24 hr period.

Rates of erythropoiesis were originally estimated by reticulocyte levels, the histologic appearance of erythropoietic tissue on the 4th post-irradiation day and iron incorporation. Since there was an excellent correlation between these measures(2), subsequent estimates of red cell production were based on Fe incorporation alone. For this determination, Fe^{59} tagged normal plasma was injected on the 2nd post-irradiation day and 48 hours thereafter blood was collected by cardiac puncture for determination of Fe^{59} activity. The details of tagging of the plasma, determination of radioactivity, and the calculation of the % Fe incorporation have been given previously(2). At the completion of each experiment, the tails of the injected rats were checked for radioactivity. The rare animals in which more than 5% of the injected Fe^{59} was present in the tail, indicating substantial extravascular injection, were excluded from the series. Rats with pneumonia, which occurs in 2 to 5% of animals in our colony, were also excluded after verification of the diagnosis by autopsy.

Results. The average Fe^{59} incorporation in 48 hours by normal non-irradiated animals was 65%, with individual animals varying from 50-80%. The average Fe^{59} incorporation for animals receiving 160-200 r of whole body irradiation was 25% (Co^{60} source) and 30% (2.5 MEV source) with a range of 10-45%. The injection of normal plasma following irradiation produced a variable response. In 3 of 4 experiments, where animals receiving normal plasma were directly compared with irradiated controls (Table I), the Fe^{59} incorporation of those receiving normal plasma was greater than in the irradiated controls. In only one of these, however, was the difference significant (Exp. 6, Table I, $p < .02$). The variability within each group receiving normal plasma was greater than that observed with control animals or those receiving anemic plasma (Table I).

The injection of plasma from anemic don-

TABLE II. Studies on Stability of Erythropoietic Factor in Anemic Plasma.

Normal plasma	Anemic plasma*				
	Fresh	Stored		Dialyzed	Heated
		4°	−20°		
38 ± 5 (7)†	53 ± 2.7 (8)	44 ± 5 (7)	51 ± 6 (5)		
39 ± 6.6 (7)	44 ± 2.7 (8)			44 ± 2.5 (5)	34 ± 5.7 (4)

* Plasma collected 24 hr after production of post-hemorrhagic anemia.

† Fe^{59} values given represent % of total injected dose present in circulating red cells 48 hr after injection ± a single stand. error computed as $\frac{\text{Range}}{K_n\sqrt{n}}$(10). Number of animals in each group is included in parentheses.

ors into irradiated animals produced a significant increase in the rate of erythropoiesis as measured by Fe^{59} incorporation (Table I). In all experiments to date the average Fe^{59} incorporation values were greater in the animals injected with anemic plasma than in those receiving normal plasma or in untreated irradiated controls. The difference in the means of groups receiving anemic and normal plasma varied from 5-32% (Table I). In 4 of 6 experiments the differences in mean Fe^{59} incorporation between animals receiving anemic and normal plasma were significant at a 0.01 level, and in one at a 0.05 level (Table I). In the remaining experiment, the mean for anemic plasma was greater than for normal plasma, but the difference was not statistically significant ($p > 0.05$). There was marked variation in the response between experiments but the variability within a given experimental group was relatively small (S.E. 1.3-4).

In one experiment anemic plasma collected 24 hours after the donor animals were bled produced a greater response than that collected 48 hours after bleeding (Table I). The factor present in anemic plasma retained its activity during storage for 3 weeks at −20°C (Table II). The supernatant obtained after heating at 100°C at pH 5.4 for 10 minutes was inactive (Table II). There was no reduction in the activity of anemic plasma following saline dialysis at 4°C for 24 hours (Table II).

Discussion. It has been suggested that the modification by acute blood loss of the usual irradiation injury to erythropoiesis is due to the elaboration of hemopoietine which accelerates the regeneration of erythropoietic tissue. This interpretation has been supported by the observation that plasma collected from anemic donors, when injected into rats immediately following sublethal irradiation, stimulated erythropoiesis as evidenced by an increase in mean Fe^{59} incorporation of 5-30% above that of animals receiving normal plasma. As might be expected, the magnitude of response in animals receiving anemic plasma was not as great as had been noted previously in animals with acute blood loss following irradiation. Although there was a considerable variation in the degree of response between experiments, all groups receiving anemic plasma had a mean Fe^{59} incorporation greater than that observed following the administration of normal plasma. In 4 experiments the differences were significant at the 0.01 level, in 1 at the 0.05 level and in 1 there was no significant difference ($p > .05$). The response of the sublethally irradiated animals to anemic plasma, therefore, appears to be highly significant. The stability of the factor present in anemic plasma was similar to that reported by Erslev (8).

The variability of response to anemic plasma between experiments may be accounted for in part by differences in the plasma content of hemopoietine. The marked difference between the experiment in which a Co^{60} source was used and those with the 2.5 MEV source, suggests that irradiation factors may account for some of the variability.

Normal plasma produced an increase in Fe^{59} incorporation in 3 of 4 experiments where there was a direct comparison with irradiated controls but in only one was the difference statistically significant. This effect may have resulted from the presence of small amounts of the erythropoietic substance in normal plasma or from a transient dilution anemia. The marked variability within groups receiving pooled normal plasma (S.E. of the mean 2.6-7.8) is thought to result from variations in responsiveness of individual animals to borderline amounts of hemopoietine.

It is of interest that only a single injection of anemic plasma is required to demonstrate erythropoietic activity in the sublethally irradiated animals, whereas multiple injections are required in normal animals(4,6). It has previously been observed that a single injection of PAPP, which fails to produce a significant alteration of erythropoiesis in the normal rat(9), will increase red cell production when administered to rats following a sublethal dose of irradiation (120-200 r from a 3 MEV source, 200 r from a Co^{60} source)(2). These observations suggest that immediately following irradiation an animal is more sensitive to erythropoietic stimuli than normally, provided that the dose of irradiation is not sufficient to destroy the majority of the erythropoietic tissue. It is tempting to suggest that the increased sensitivity of the irradiated animal results from the destruction of an inhibitor thus altering the normal balance between inhibitors and stimulators of erythropoiesis. The sublethally irradiated rat may serve as a useful animal for the further investigation of the regulation of erythropoiesis, since the method employed requires but a single treatment, allows a satisfactory estimate of red cell production by the Fe^{59} incorporation technic and requires only 5 days for an experiment. At present, the method requires the use of 5-7 animals per group and the inclusion of controls. The variability of response may be in part a reflection of the plasma content of hemopoietine rather than the lack of sensitivity of the test.

Summary. Injection of plasma from anemic donors into rats immediately following exposure to sublethal doses of X-rays produced a statistically significant increase in erythropoiesis as measured by Fe^{59} incorporation. The factor present in anemic plasma was nondialyzable and was not present in the supernatant obtained after precipitation of protein by heating. Normal plasma produced a variable response. Use of sublethally irradiated rat as a test animal in the study of regulation of erythropoiesis is suggested.

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Received November 14, 1955. P.S.E.B.M., 1956, v91.

Absorption and Elimination of $Carbon^{14}$ Labelled Nicarbazin by Chickens.
(22153)

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Nicarbazin, an equimolecular complex of 4, 4′-dinitrocarbanilide (DNC) and 2-hydroxy-4, 6-dimethylpyrimidine (HDP), is effective for prevention of coccidiosis in chickens(1). This complex is split into its components in the animal, and the HDP portion rapidly eliminated. By use of spectrophotometric methods, both moieties of nicarbazin have been detected in tissues of chickens receiving the drug(2). However, 2 days after

TABLE I. DNC in Chicken Tissues following the Oral Administration of Nicarbazin.

Tissue	Time after final dose (hr)	DNC and metabolites, γ/g tissue calculated as DNC	DNC (colorimetric), γ/g tissue	Ratio: Radioactive/Colorimetric
Plasma	3	4.4	3.8	1.2
	24	7.2	4.9	1.5
Liver	3	41.8	32.0	1.3
	24	67.8	33.0	2.0
Thigh muscle	3	9.7	4.8	2.0
	24	11.3	4.6	2.5
Breast muscle	3	6.8	4.7	1.4
	24	9.9	2.9	3.5

withdrawal of nicarbazin from the diet, neither DNC nor HDP could be detected in the tissues by chemical methods.

There was the possibility, however, that metabolites of DNC undetected by the chemical means used might remain in the tissues. Therefore, nicarbazin labelled with C^{14} in the benzene ring of DNC was prepared and fed to chickens. The data show that after discontinuance of treatment, both DNC and its metabolites are rapidly eliminated from the tissues.

Exp. 1. Two 200 g chickens were given oral doses of labelled nicarbazin (activity, 0.05 μc/mg)* suspended in 2% methyl cellulose solution. Each bird received 3 doses of 126 mg at 3 hour intervals. One chicken was sacrificed 3 hours, the other 24 hours after the final dose. Aliquots of aqueous tissue homogenates were dried at room temperature in stainless steel planchets and counted in a Q-gas flow counter. Counts were corrected for background and for self-absorption. Counting was done for sufficient time to assure statistical accuracy of 1% to 4%, depending upon activity of the sample. Colorimetric estimation of DNC in the homogenates was by the method previously described(*2*).

Results. The ratio of apparent DNC, determined by radioactivity measurements, to that found by the chemical method ranged from 1.2 to 3.5 (Table I). It is evident that DNC metabolites were present in the tissues. Although tissue samples taken 3 and 24 hours after dosing were from different chicks, the data suggested that during the 24-hour period, some accumulation of DNC metabolites had occurred.

Exp. 2. Groups of 12 chickens (one-week-old New Hampshire Reds, weighing 70-84 g) were fed diets containing 0.015% or 0.06% labelled nicarbazin. Radioactivity measurements were made on blood taken daily from 6 chickens of each group, each group of 6 being bled on alternate days. On the seventh day, 3 birds from each group were sacrificed. The remaining chicks were placed on stock rations, and 3 from each group sacrificed 1, 2

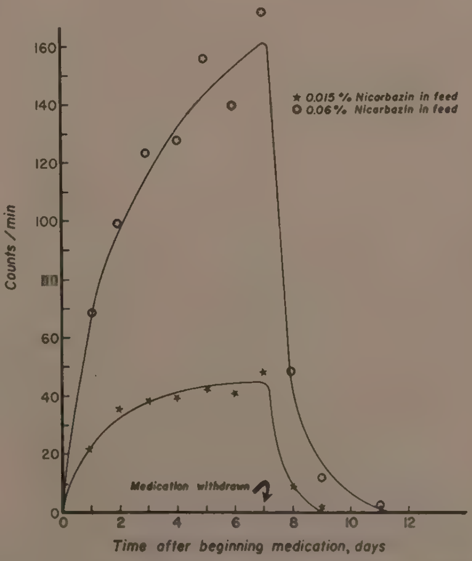

FIG. 1. Radioactivity in blood of chickens during and after medication with C^{14}-labeled nicarbazin.

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* We are indebted to Dr. Elbert Harris, Chemical Division, Merck & Co., Inc., who made the material available to us.

TABLE II. DNC in Chicken Tissues after the Feeding of Nicarbazin in the Diet.

Days after medication discontinued	0	1	2	4
Tissue	DNC, γ/g wet tissue or γ/ml blood plasma			
	0.015% nicarbazin in feed			
Thigh muscle				
Radio.	1.8	.5	.35	.32
Chem.	4.1	.0	.0	—
Breast muscle				
Radio.	2.1	.6	.0	.03
Chem.	1.7	.0	.0	—
Kidney				
Radio.	9.0	.2	.05	.0
Chem.	5.5	.0	.0	—
Liver				
Radio.	14.5	3.8	.54	.01
Chem.	8.0	1.0	.0	—
Blood				
Radio.	2.7	.5	.03	.05
Chem.	1.8	.1	.0	—
	0.06% nicarbazin in feed			
Thigh muscle				
Radio.	12.1	3.3	2.0	.19
Chem.	14.3	1.9	.4	—
Breast muscle				
Radio.	7.4	2.4	.4	.24
Chem.	7.2	2.0	.0	—
Kidney				
Radio.	31.5	1.1	.17	.0
Chem.	25.7	2.9	.0	—
Liver				
Radio.	25.7	11.3	3.6	.24
Chem.	15.6	9.6	.4	—
Blood				
Radio.	9.6	2.8	.75	.14
Chem.	7.0	1.4	.1	—

and 4 days later. Tissues and blood were assayed as in Exp. 1.

Results. Radioactivity in blood of chickens fed 0.015% nicarbazin (Fig. 1) approached a plateau in the 7-day feeding period. The slope of the curve relating radioactivity to time for birds fed 0.06% nicarbazin, decreased with time. If a longer feeding period had been used, it is probable that this curve also would have approached a plateau. Withdrawal of medication was followed by a precipitous fall in radioactivity to zero in 2 to 4 days.

Radioactive counts and chemical analyses of the tissues (Table II) agreed in demonstrating that two days after withdrawal of 0.015% medication, and 4 days after withdrawal of 0.06% medication, only traces of DNC or its metabolites were present in the tissues.

Summary. Tissues of chickens fed diets containing 0.015% and 0.06% radioactive nicarbazin for 7 days contained DNC and minimal quantities of its metabolites. Two and four days, respectively, after withdrawal of 0.015% and 0.06% medication, only traces of DNC and its metabolites remained in the tissues.

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Hormone Content of Anterior Pituitary of Monkey (*Macaca mulatta*)* with Special Reference to Gonadotrophins. (22154)

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Pituitaries from monkeys, of both sexes and of different ages, have been assayed in hypophysectomized rats for their content of gonadotrophic hormones. These studies were undertaken to determine any peculiarities either in quantity or proportion of the gonadotrophic hormones, preliminary to treatment of macaques with preparations from homolo-

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* This investigation has been made with the assistance of a grant from the Committee on Research, Council on Pharmacy and Chemistry, Am. Med. Assn., and grants from U. S. Public Health Services.

gous anterior pituitaries. Estimates of levels of other pituitary trophic hormones were made concurrently.

Methods. Pituitaries were removed from adult female monkeys at representative stages of the menstrual cycle, and from adult and immature males, for assay of hormone content of individual glands.[†] Pituitaries were also collected from large numbers of males and females of variable ages, and pooled for assay and future chemical fractionation. The anterior lobes were dissected free from dura and from posterior and intermediate lobes. Anterior pituitaries from individual animals were frozen at once and lyophilized; pooled anterior lobes were frozen within 2 hours after sacrifice. Wet and dry weights of individually collected anterior pituitaries were determined, and average weight in pooled lots could be calculated from the collective wet or dry weight and the number of glands. Assays were performed in immature female rats, hypophysectomized at 26-28 days of age, beginning 6 to 8 days after operation. Groups of 3 rats were used for each dose level. No data from incompletely hypophysectomized animals have been included. The postoperative period of one week used in most experiments was selected as fulfilling a condition defined in assay of gonadotrophins, follicle stimulating hormone (FSH) and interstitial cell stimulating hormone (ICSH). The approximate titer of the other trophic hormones of the pituitary, growth hormone (GH), adrenocorticotrophic hormone (ACTH), and thyrotrophic hormone

TABLE I. Hormone Assay of Anterior Pituitaries of the Monkey. MED expressed as total dose in dry weight (mg).

Donors					Wt (mg) anterior lobe		Assay in hypophysectomized rats (mg)				
	Age										
Sex		Cycle day	Wt, kg	Test conditions	Wet	Dry	FSH	ICSH	ACTH	TSH	GH
Females	Adult	1	5.4	Subcut.: FSH, ACTH, TSH	59	13.2	1.0	.25	1.5	1.0	.25
		1	5.0	IP: ICSH, GH	58	12.5	1.5	.75	2.5	1.0	.08
		9	4.2	Daily, 3 da; 72 hr	43	9.8	.25	.1	>1.5	.18	.08
		9	6.8		48	11.9	.5	.15	1.0	>1.5	.05
		11	6.4		57	12.8	1.0	.1	>1.5	>1.5	.08
		15	4.5		46	10.0	2.5	.25	2.0	.25	.30
		22	4.3		42	9.6	1.5	.5	1.6	.3	.08
	Immature (estrin)		2.1	As above		6.7	>1.0	>1.0	>1.0	>1.0	.10
			2.1			6.3	>1.0	>1.0	.5	.5	.10
Males	Adult		8.2	As above	36	9.7	.75	.3	>2.0	1.5	.08
	Immature		3.0	One implant; 96 hr	24	6.0*	>3.0	>3.0	1.5	<1.5	<1.5
			3.4	*Idem*	20	5.0*	>2.5	>2.5	>2.5	1.2	<1.2
♂ & ♀ pooled	Variable ages			Subcut. 4 da; 96 hr	13.9	3.2	3.2	3.2	6.4	.8	< .8

* Implanted fresh; approximate dry wt derived by factor of 4.

[†] Pituitaries of adult female monkeys in known stages of cycle were obtained from the Yale Obstetrics Colony. Pituitaries of immature male monkeys used to make implants of individual glands were obtained from the Virus Laboratory, University of California, through the courtesy of Dr. Mary McClain. The large numbers of glands which were pooled, frozen and dried, were obtained from the Cutter Laboratories through the kindness of Dr. Walter E. Ward, Dr. Donald Trotter and Mr. Donald H. Wonder. Pituitaries from estrinized monkeys (450 μg estradiol benzoate over a period of one week previous to autopsy) were obtained through the courtesy of Dr. H. R. Catchpole, University of Illinois School of Medicine.

TABLE II. Hormone Content of Anterior Pituitaries of the Monkey.

Donors			Total units per anterior lobe				
	Age						
Sex		Cycle day	FSH	ICSH	ACTH	TSH	GH
Females	Adult	1	13	53	9	13	53
		1	8	17	5	13	150
		9	40	100	<7	54	120
		9	24	80	12	<8	240
		11	13	130	<9	<9	160
		15	4	40	5	40	30
		22	6	19	6	30	120
	Immature (estrin)		<7	<7	<7	<7	67
			<7	<7	13	13	63
Males	Adult		13	32	<5	5	120
	Immature		<2	<2	4	>4	>4
			<2	<2	≤2	4	>5
♂ & ♀ pooled	Variable ages		≥1	≥1	.5	>4	4

(TSH), could also be determined even though the postoperative period was too short to allow for an entirely satisfactory state of regression of the target organs.

Results. Potency of anterior pituitaries from various types of monkeys is expressed in Table I as the minimal effective dose (MED) or unit value for each of the 5 principal trophic hormones.[‡] Total hormone content expressed in units per anterior lobe is given in Table II.

Pituitaries of Normal Adult Females. Anterior lobes from adult female monkeys sacrificed at known stages of menstrual cycle, were pulverized, suspended in saline and injected daily for 3 days. The MED for FSH ranged from 2.5 to 0.25 mg dry weight of gland; thus the total hormone per anterior lobe varied between 4 and 40 units. The titer of FSH was highest on ninth day of cycle, the MED being 0.5 to 0.25 mg; 24 to 40 units were therefore present per gland. In early and late periods of the cycle the MED varied from 2.5 to 1.0 mg, with 4 to 13 units present per gland.

Unitage of ICSH was higher than that of FSH. The MED varied from 0.75 to 0.1 mg and total content ranged from 17 to 130 units per gland. Potency was highest between days 9 and 11, MED being at this time about 0.1 mg with 80 to 130 units present per gland. The content of ICSH increased relatively more than that of FSH during middle of the cycle, so that the ratio of ICSH to FSH increased above its usual value of 3 to 1, and reached a value of 10 to 1 on days 11 and 15. Evidence from the response of hypophysectomized recipients is not conclusive that monkey pituitary at any specific period in the cycle contained the optimum ratio for corpus luteum formation. It is to be noted, however, that corpus luteum formation with ovulation was induced by a total dose of 1.5 mg of pituitary tissue taken during first half of menstrual cycle (days 1, 9 and 11), whereas even higher doses of pituitaries, removed during the last half of the cycle (days 15 and 22), resulted only in minimal follicular stimulation in hypophysectomized rats. Growth hormone was present in considerable unitage in the adult female pituitaries, demonstrable at 0.3 to 0.05 mg total dose. There was no significant change in minimal effective dose or in total pituitary content during the cycle, more than 100 units per pituitary usually being demonstrable. The adrenocorticotrophic hormone content was consistently low, the hormone frequently detectable by change in lipid pattern in the adrenal of the recipient only at the highest levels tested, 2.5 or 1.5 mg. At most, 5 to 10 units were present per anterior pituitary. The minimal effective dose for thyrotrophic hormone was usually between 1.0 and 0.2 mg, total unitage ranging between 10 to 50 units per pituitary, with no cyclic pattern.

[‡] MED for *follicle stimulating hormone* is defined as the minimal total dose which will cause microscopically detectable growth of ovarian follicles beyond control level, specifically to the stage of beginning antrum formation. The MED for *interstitial cell stimulating hormone* is the total dose which will cause microscopic evidence of beginning repair of the "deficient" type of interstitial tissue, including enlargement of nuclei, lighter nuclear stain and reappearance of a rim of eosinophilic cytoplasm. The MED for *thyrotrophic hormone* is the total dose which causes increase in height of epithelium, from squamous to low cuboidal, with beginning vacuolation of colloid. MED for *growth hormone* is total dose which will cause an increase of 30 to 50 micra in width of uncalcified portion of proximal epiphyseal cartilage of tibia stained with silver nitrate. MED for *adrenocorticotrophic hormone* is total dose which causes beginning repair of adrenal cortex as seen in frozen sections stained with Sudan black; the criteria include replacement of coarse by fine lipid droplets, infiltration of lipid into the subglomerular lipid-free zone, increase in cell size and width of cortex, and regeneration of the zona reticularis.

Pituitary of adult male. The anterior pituitary from an adult male macaque was assayed as above. The weight of anterior pituitary was about the same as the smallest from the normal adult females. Unitages of various hormones were roughly within the same range as in normal adult female pituitaries, though the content of FSH did not equal that reached in the female pituitary in the middle of the cycle. The total amount of ICSH present per anterior pituitary was within the lower range found in the normal female. The ratio between total content of ICSH and FSH (2 to 1) was as low as any encountered in the female pituitary, either at beginning or end of the cycle. Ovulation and corpus luteum formation in hypophysectomized rats resulted from total doses of 2 mg. The content of GH was comparable to that in the adult female, that of TSH and ACTH was even lower than in normal females.

Pituitaries of immature males. Assays of pituitaries of immature males were not strictly comparable to those of the normal adult male pituitary just cited, inasmuch as repeated injections were not given but fresh anterior lobe tissue was implanted. The content of all hormones, notably that of gonadotrophic hormones, appeared to be lower than in adult pituitaries.

Pooled pituitaries of monkeys. These pituitaries were collected from male and female monkeys of unknown age, though predominantly from young animals. The frozen-dried pituitaries were injected in saline suspension daily for 4 days. The hormone content per pituitary was lower than that in the individual adult female or male pituitaries but was within the same range as in individual immature male pituitaries.

Pituitaries of estrinized immature females. Pituitaries removed from 2 females which had received estradiol benzoate were lyophilized and assayed individually as above. The pituitaries were small. Neither FSH nor ICSH could be detected, even after injection of much higher doses than were required for normal adult female or for pooled pituitaries. The content of ACTH, GH and TSH was in the low normal range.

Discussion. Very few accounts have been found in the literature pertaining to the content of gonadotrophic or other trophic hormones in monkey pituitary. Salhanick, Hisaw and Zarrow(1) examined gonadotrophic potency of pituitaries of castrate macaques and of castrates treated with estradiol. Gonadotrophic potency of the castrate pituitary was high and was depleted by estrogen therapy; no value was given for hormone content in the normal animal. As stated in the present paper, gonadotrophins could not be detected, even at high doses, after estradiol treatment of immature females.

The hormone content of human pituitaries has received more attention. Some studies which should be mentioned are those of Witschi and Riley(2), Bruner(3), Bahn, Lorenz, Bennett and Albert(4-7), Leathem(8), and Burt and Velardo(9). Assays conducted by Witschi and Riley, and by Bahn *et al.*, have probably been the most comprehensive, including assays of pituitaries of normal adult men and women, of postmenopausal and pregnant women, of old men and of children. Those of Bahn and collaborators are particularly significant for comparison with our results for monkey pituitaries, because the same type of assay animal (the hypophysectomized rat) and similar morphological criteria were used in establishing unitage. Furthermore, these authors assayed anterior lobe tissue free of posterior lobe so that their estimates of hormone content can be compared directly with those given here. A total dose of 3 mg of dry anterior pituitary tissue from women was the minimal amount necessary for detection of either FSH or ICSH. In the adult female monkey the minimal amount needed for demonstration of FSH ranged from 2.5 to 0.25 mg, and of ICSH from 0.75 to 0.1 mg, both varying with the menstrual cycle. The ratio between minimal effective doses for ICSH and FSH was 2 or 3 to 1 at beginning and end of the cycle and rose to 10 to 1 on days 11 and 15, whereas a ratio of unity was reported for the human pituitary. Bahn *et al.* found that the MED for gonadotrophic hormones was the same in men as in women, the ratio of ICSH to FSH again being unity. The values given here for minimal effective doses of adult male monkey pituitary were lower; ICSH 0.3 mg, FSH 0.75 mg, and the ratio was 2.5 to 1. Both Bahn *et al.*,(6) and Witschi and Riley(2) have found a low gonadotrophic content in pituitaries of children, the content being 1/5 to 1/10 that in adult pituitaries. The gonadotrophic hormone content of pituitaries of immature monkeys recorded here was correspondingly low.

The assays of monkey pituitaries were made to determine whether their gonadotrophins were peculiar either qualitatively or quantitatively, in the hope that such data might guide efforts to induce sexual maturation and ovulation in monkeys. All pituitary preparations hitherto injected in such experiments have been from heterologous pituitaries (chiefly from pig and sheep). The gonadotrophic potency of rhesus pituitaries was higher than that of sheep pituitaries. The MED for both FSH and for ICSH in adult female monkey pituitaries at the time of highest hormone content was 1/10 that for sheep pituitary(10). The two gonadotrophic hormones were present, however, in the same proportion in sheep and monkey pituitaries. In acetone dried sheep pituitaries the MED for ICSH was 1 mg, for FSH 2.5 mg; in lyophilized monkey anterior lobe, the MED for ICSH was 0.1 mg, for FSH 0.25 mg.

In the monkey anterior pituitary the content of FSH was highest on 9th day of menstrual cycle, compatible with the supposed preovulatory spurt of follicular growth at about this time. The content of ICSH was highest in preovulatory and ovulatory period, consistent with the synergistic action between ICSH and FSH, and its probable role in final follicular enlargement, and also with the importance of ICSH in corpus luteum formation. The ratio of ICSH to FSH was highest in ovulatory and early postovulatory period. However, pituitaries from female monkeys in the first half of the cycle, as well as those from the adult male, caused ovulation in hypophysectomized recipients, even though in both types of pituitaries the ratio of ICSH to FSH was low. Failure of pituitaries from female monkeys in the last half of the cycle to induce ovulation must have been due to low unitage, particularly of FSH.

Summary. Individual anterior pituitaries as well as pooled glands of *Macaca mulatta* have been assayed in hypophysectomized immature female rats for their content of trophic hormones, particularly gonadotrophic hormones. 1. In the adult female macaque, the content of follicle stimulating hormone was highest between the ninth and eleventh days of the cycle. The content of interstitial cell stimulating hormone was also maximum at this time of cycle. The ratio of ICSH to FSH was highest (10 to 1) on days 11 and 15. 2. In adult male anterior pituitary the total content of ICSH and FSH and the ratio between these hormones was approximately the same as in the adult female pituitaries at beginning and end of menstrual cycle. The gonadotrophic hormone content of pituitaries of immature animals was lower than in adults. 3. Other trophic hormones in the adult female pituitary, thyrotrophic, adrenocorticotrophic and growth hormones, did not change in amount during the menstrual cycle. In adult male, as well as in the female pituitary, thyrotrophic and adrenocorticotrophic hormones were present in low concentration, but growth hormone was present in high unitage.

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Determination of Evans Blue Dye in Blood and Tissues.* (22155)

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To our knowledge there is only one report in the literature of a method for determining Evans blue dye in whole blood(1) and only 3 for determining the dye in tissues(2-6). The present paper describes a new procedure for its estimation in tissues, of which blood may be considered a special case. In addition, by employing this method, data have been obtained on the fate of the dye for up to 140 hours after its injection into the rat.

Method. Description of method. The method is based upon 3 fundamental operations: (1) homogenization of tissue in a concentrated solution of urea, (2) splitting of the Evans blue-protein complex and partial precipitation of chromogens with acetone, and (3) complete precipitation of chromogens by addition of Somogyi reagents. Details of the method as employed for tissues are as follows: (1) A 5-g sample of the dye-containing tissue to be analyzed and 2 additional 5-g dye-free samples of similar tissue obtained from another animal were weighed. To one of the latter was added a known amount of dye in approximately the amount to be found in the unknown tissue sample. The 3 samples (tissue plus unknown dye, tissue plus known dye, tissue without dye) were minced. (2) Concentrated urea solution (equal weights of A.R. urea and distilled water) was added to each tissue sample until the total volume was 100 ml and the mixture was homogenized in a Waring Blendor for 10 minutes. The homogenate was poured into a flask in a cold tap water bath and allowed to cool for 5 minutes. (3) Two hundred ml of A.R. acetone added to each sample and each was shaken briefly. Ten ml of Somogyi reagent I (10% zinc sulfate • $7H_2O$) was added to each sample which was then mixed well by shaking. After further addition of 10 ml of Somogyi reagent II (0.5 N NaOH) to samples, the latter were mechanically shaken for 10 minutes and allowed to stand 15 minutes. (The 2 Somogyi reagents should be standardized by titration against each other to the end point of phenolphthalein). (4) The samples were filtered through Whatman No. 42 paper. Optical density of filtrates from the 2 dye-containing samples was determined at 620 mmu in colorimeter which has a 20 cm cell length. The tube containing dye-free tissue was used for the I_o setting. *Procedure for analysis of blood.* The method was applied to blood as follows: (1) one-half to 2 ml of whole blood was taken and 0.9% NaCl was added where necessary to bring the final volume to 2 ml. Two ml of the urea solution used for tissue analyses was added followed by 8 ml of A.R. acetone and 0.5 ml each of the 2 Somogyi reagents. The samples were shaken between each addition. (2) After centrifugation at high speed for 15 minutes the supernatant liquid was taken for colorimetry at 620 mmu.

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TABLE I. Recovery of Added Evans Blue from Dog Tissues.

Tube No.	Contains			Optical density	% yield
I_0	Water only			.0	0
1	All reagents (a check on water as I_0)			.0	0
2	Dye + reagents (except Somogyi reagents)			.218	100
3	Heart muscle, no dye			.002	1
4	"	" +	"	.222	102
5	Intestine,	no	"	.0	0
6	"	+	"	.218	100
7	Brain,	no	"	.002	1
8	"	+	"	.215	98.5
9	Liver,	no	"	−.002	−1
10	"	+	"	.215	98.5
11	Kidney,	no	"	.002	1
12	"	+	"	.218	100

The standard solution was made with known amounts of dye added to an amount of whole blood equal to that taken for analysis of the unknown. The solution used for the I_0 setting was made from dye-free blood. With less than 0.5 ml of blood the optical density is a function of amount of blood because of the effect of blood colloids and salts on absorption of light by the dye. *Fate of dye in rats.* 1. While under sodium pentobarbital (Nembutal) anesthesia each of 8 fasted rats was injected with 0.25 ml of 5% Evans blue dye into the exposed right jugular vein. Twenty minutes later was taken as zero time, and concentration of dye in a 0.2-ml sample of tail blood at this time was taken as 100%. The animals recovered from the anesthetic 4 to 8 hours later. The animals sacrificed within 16 hours were placed in cages without food but with water. The animals sacrificed after 16 hours were placed in cages with food and water but were taken off food for 12 hours before they were sacrificed. At various time intervals up to 140 hours individual rats were stunned by blow on head and 0.2 ml blood was taken from the still beating heart. Blood and whole rat carcass were analyzed for dye by methods previously described using a 6% correction for weight loss of rat tissues caused by evaporation of water during grinding. Excreta were included in the carcass analysis only in the case of rats sacrificed before recovery from anesthesia. To judge from color of urine and feces, their dye content was relatively trivial.

Results. Recovery of dye from various tissues. Two experiments were run, each on a set of 5 different tissues obtained from dogs. In the first experiment 0.0376 mg of Evans blue was added per gram of experimental tissue and a colorimeter equipped with a 20 cm cuvette was used. In the second experiment 0.10 mg of dye was added per gram of tissue, and the Coleman Spectrophotometer equipped with a 1 cm cuvette was employed. Since data from both experiments were essentially the same, only those from the second experiment (0.1 mg dye per g of tissue) are given (Table I). Recoveries of added dye ranged from 98-102%. Blood was added to dye-containing tissue samples to determine the contribution of blood pigments to non-dye color in the final solution. The results (Table II) indicate that added blood up to 50% of weight of the tissue sample does not result in a positive error. More blood than this yields erroneously high dye recoveries.

Recovery of injected dye by analysis of the whole animal. Evans blue was administered intravenously to each of 14 rats and the whole

TABLE II. Effect of Added Blood on Dye Recovery from Tissues.

Sample	g of tissue	ml of blood added	% yield
Control	0	0	100.0
1	5	0	102.5
2	5	2.5	100.0
3	5	5.0	106.5
4	5	7.5	111.0
5	5	10.0	117.5

TABLE III. Assay of Whole Rat for Evans Blue Content.

Sample	Optical density	% yield on basis of raw data	% yield corrected for wt loss during grinding
Rat	.444	102.8	98.8
Control	.432	100.0	—

animal was taken for analysis. The following is a typical protocol: A rat weighing 409 g was fasted overnight and anesthetized wtih sodium pentobarbital (Nembutal), 35 mg/kg given intraperitoneally. One ml of 1.0% Evans blue dye was injected into the exposed jugular vein. It was necessary to fast the animals because fox chow in the gastrointestinal tract contributed errors of 7 to 28% due to a green color which is extractable from the chow and which appears in the final solution used for colorimetry. One ml of the same 1% dye solution was added to 409 ml of water to serve as a standard. The rat was killed by a blow on the head following which it was quartered and ground in a meat grinder 6 times. The tissue mass was blended with spatulas between the several grindings. After grinding, the tissue weighed 393.6 g, a loss of 15 g from evaporation of water. The final result was corrected for this weight loss. Five g of the tissue were taken for analysis. A second rat which received no dye was ground in the same manner and 5 g of its tissues were added to 5 ml of the standard solution. Another 5 g of tissue from the second rat was employed for preparation of the solution used for the I_o setting. All samples were homogenized in concentrated urea solution and treated with acetone and the Somogyi reagents as described above. Colorimetry was done at 620 mmu using a colorimeter equipped with a 20 cm cuvette. The results are given in Table III. In Table IV are given the results for the remaining 13 rats. In this series the method was accurate within 3% of the true value. In preliminary trials, proportions of urea solution and tissues, amounts of the 2 Somogyi reagents and the amount of acetone were varied independently. The method as specified is based upon optimal reagent amounts as established in these trials.† It was necessary to add dye-free tissue to the standard dye sample to prevent the shift of dye color from dark blue to light purple, as may occur in acetone-water solutions in the presence of Somogyi reagents.

TABLE IV. Recovery of Added Evans Blue from Whole Rats.

Rat No.	% yield (corrected)*
1	103.2
2	97.7
3	97.1
4	97.5
5	102.5
6	101.1
7	101.2
8	103.2
9	101.0
10	97.1
11	99.5
12	98.6
13	102.9
Rat from Table II	98.8

* For wt loss during grinding.

Fate of injected dye in rats. The data are presented in Fig. 1, in which the lines were fitted to the plotted points by visual inspection. Because of this and because each point for a given time interval was obtained from a single animal, only the general features of the curves may be considered as meaningful. Furthermore, the points for percentage of dye in the body which is extravascular have been calculated on the assumption that blood volume has remained constant, *i.e.*, that the amount of intravascular dye is directly proportional to blood dye concentration. For large changes in blood dye concentration, errors introduced by this assumption may be considered of secondary importance. Proceeding on the basis of this assumption, one finds that 40 hours after its administration, about 90% of the dye has left the blood but approximately 83% is still in the whole carcass. Even after 140 hours, when about 92% has left the blood, some 67% can be still recovered from the carcass. Disappearance of the dye from the body is most rapid 10-24 hours after injection, and is attributed mainly to decolorization within the body rather than loss via the excreta. Thus most of the dye which presumably disappears from blood in colored form can be recovered from the whole animal even at 140 hours. This finding might be ex-

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† Details of these trials are available elsewhere(8).

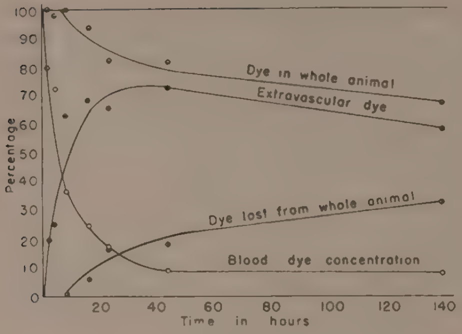

FIG. 1.

plained by the presence in blood of material which yields color by carcass analysis but not by blood analysis. This is highly improbable and, moreover, no blue color was observed in Somogyi precipitates of blood. The conclusion appears justified that the dye in the carcass not accounted for by blood analysis is in the extravascular spaces.

Discussion. Sellers *et al.*(7) have reported serum Evans blue/serum protein concentration ratio as a function of time up to 70 hours after intravenous injection into rats of 20 mg of Evans blue. They make no mention of employing anesthesia except for terminal sampling. If serum protein concentration is assumed to remain constant, their curve can be considered a blood dye concentration curve, and in fact their curve is almost identical with that for blood in Fig. 1. This similarity suggests that the present findings were not greatly influenced by the ether anesthesia.

Summary. 1. A method is described for determination of Evans blue dye in various animal tissues. In brief it consists of (1) homogenization of tissue in a concentrated solution of urea, (2) splitting of the Evans blue-protein complex and partial precipitation of protein and chromogens with acetone, and (3) complete precipitation of chromogens by addition of Somogyi reagents. 2. The method has been employed to follow the fate of the dye after intravenous injection into rats. After such administration of 12.5 mg of Evans blue, about 90% of the injected dye apparently left the blood but only about 15% could not be recovered from the body in 40 hours. At 140 hours after injection about 67% of the injected dye could still be recovered from the body.

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Plasma Prothrombin Time and Hematocrit Values of Blood of Dairy Cattle.* (22156)

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Hemostasis is a complicated mechanism of blood coagulation composed of many coordinated reactions(1-3). According to Quick (2), coagulation of blood occurs in 3 stages, none of which are complete when considered individually. These stages must be combined in a definite arrangement before a complete explanation of hemostatic mechanism can be offered.

Determination of plasma prothrombin time

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has become essential in diagnosing, treating, and studying hemorrhagic diseases in humans (4). Since prothrombin level of blood in animals is decreased by dicumarol present in damaged sweet clover(5), in avitaminosis-K (6), and in other hemorrhagic diseases(7,8), normal prothrombin time is of considerable value in veterinary medicine. Pritchard *et al.* (9) found clotting time of blood to be lengthened when soybeans extracted with trichloroethylene were fed to cattle, but plasma prothrombin time was not lengthened appreciably. A direct correlation between plasma prothrombin time and blood clotting time was not found. This emphasizes the need for both plasma prothrombin time and blood clotting time in research with hemorrhagic diseases. A normal prothrombin time (adequate prothrombin in blood) is possible with prolonged clotting time. In this case it appears that deficiency of any one of the items (labile factor, fibrinogen, calcium, or thromboplastinogen) described by Quick(2) in blood coagulation may be present. On the other hand, prothrombin time may be prolonged with normal coagulation time. Normally excess of prothrombin is present in plasma; therefore hypoprothrombinemia must be marked before coagulation time is lengthened. Prothrombin time of plasma of sheep has been reported (10). There is, however, little information available on other ruminants. Prothrombin time of cattle is herein reported.

TABLE I. Average Plasma Prothrombin Time Values of Matured Cows.

Breed	Herd No.	No. animals	Prothrombin time (sec.)
Jersey	1	8	20.63 ± 2.33*
	2	10	20.60 ± 3.43
	3	18	25.83 ± 2.54
	4	22	22.77 ± 3.78
	5	8	19.25 ± 1.25
	6	23	27.04 ± 5.28
	Total	89	Avg 23.74 ± 4.61
Ayrshire	5	9	20.67 ± 4.21
Guernsey	5	8	20.38 ± 2.27
Holstein	5	9	22.89 ± 2.03
All breeds	Total	115	Avg 23.20 ± 4.42

* Stand. dev.

Methods. The first phase of this investigation was made possible through cooperation of several dairy farmers. Cows had been fed a standard dairy ration in addition to pasture grazing. Sweet clover pasture or clover hay had not been fed the past year. In the second phase, calves from another experiment (11) were divided into 3 groups. Group I calves were fed a standard calf-starter mix (basal); Group II calves were fed basal ration plus 5 trace minerals (Fe, Cu, Co, Mn, and Zn); and Group III calves were fed basal ration, trace minerals, fat-soluble vitamins, water-soluble vitamins, calcium, magnesium, phosphorus, and aureomycin. Blood samples were obtained from jugular vein using a mixture of ammonium and potassium oxalate(12) as the anticoagulant. In carrying out the one-stage plasma prothrombin time of Quick(2) 0.1 ml of plasma was pipetted into 7 by 100 mm pyrex test tubes. To the test tubes containing plasma, 0.1 ml of Bactothromboplastin† was added. Blood plasma and thromboplastin were mixed and placed in a thermostatically controlled water bath at 37°C. After 5 minutes, 0.1 ml of 0.02 M calcium chloride solution was blown forcibly from a pipette into the mixture and agitated by shaking gently in water bath. A stop watch was started simultaneously with addition of calcium chloride solution. At the end of approximately 10 seconds the tube was wiped free of water and held so that its contents were between the eye and a bright source of light. By rotating the test tube clockwise, it was possible to determine immediately when the solution began to clot and adhere to the wall of the test tube. Prothrombin time reported is that interval between addition of 0.02 M calcium chloride solution to the plasma-thromboplastin mixture and the first evidence of formation of a discernible clot or gel. Hematocrits were obtained by centrifuging 1 ml of oxalated blood from cows in Wintrobe hematocrit tubes for 30 minutes at 3000 rpm(13). Results were read directly from tubes as % packed erythrocytes.

Results. Plasma prothrombin time was obtained on 115 matured dairy cows and 90 dairy calves from 2 to 24 weeks of age. Table I shows that average prothrombin time for 6 herds of Jerseys was 23.74 ± 4.61. Analysis of variance showed that there was a highly significant difference (1% level) among herds. By calculating the significant mean difference among Jersey herds, the difference between herds 3 and 4 reached the 5% level of significance, differences between herds 1 and 3 and between 2 and 3 reached 1% level, and differences between herds 1 and 6, 2 and 6, 3 and 5, 4 and 6, and 5 and 6 reached 0.1% level of significance.

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† Difco Laboratories, Detroit, Mich.

In analyzing data statistically of 4 breeds of cows in Table I, the F-value reached the 5% level of significance. The difference between the means for Jerseys and Guernseys was significant at the 5% level. The difference between Jerseys and Ayrshires approached the 5% level of significance.

According to Quick(2) plasma prothrombin time should be determined within 30 minutes after blood is collected, if accuracy is desired. A comparison was made of plasma prothrombin time values determined at 0.5, 2.5, and 3.5 hours after bleeding on 10 animals in herd 2. At these intervals prothrombin time values were 20.6 ± 3.43; 20.2 ± 3.80; and 21.0 ± 2.75, respectively. These values were of no significance statistically when compared with 0.5 hour values.

Prothrombin time was obtained on plasma samples from 20 newborn calves prior to nursing. A clot or gel could not be detected in the test tube as usually observed. These test tubes were then placed in temperature controlled water bath for 2 hours, with periodic checking for clot formation. During this 2 hour period a clot did not form.

Table II shows plasma prothrombin time values of heifer calves 2 to 24 weeks old. There was no significant difference in prothrombin time values associated with age.

Plasma prothrombin time values of bulls and heifers were pooled to evaluate breed effects involving 90 bleedings. Average prothrombin time for Holstein calves was 25.15 ± 4.63 as compared with 24.55 ± 4.35 for Ayrshires. There is no significant difference between the values for Holstein and Ayrshire calves. Evidently rations did not alter prothrombin time values of calves significantly.

TABLE II. Average Plasma Prothrombin Time Values of Holstein and Ayrshire Calves at Various Ages.

Age (wk)	No. animals		Prothrombin time (sec.)	Range (sec.)
2	11		26.91 ± 4.51*	18-32
4	11		23.00 ± 5.53	16-30
6	12		25.92 ± 4.85	22-35
8	14		25.50 ± 3.63	20-33
12	13		24.85 ± 4.21	20-34
18	8		23.38 ± 4.47	20-30
24	4		26.50 ± 4.51	18-30
Total	73	Avg	25.11 ± 4.54	16-35

* Stand. dev.

Average prothrombin time for control calves was 24.80 ± 3.88, for trace mineral supplemented calves 24.61 ± 4.23, and for the vitamin-supplemented group 24.83 ± 5.27. Trace minerals and other nutrients in addition to aureomycin did not delay or shorten prothrombin time of blood plasma.

Plasma prothrombin time values for Holstein and Ayrshire calves were pooled giving an average of 23.56 ± 3.96 seconds for bull calves and 25.10 ± 4.56 for heifers. These averages show a lower prothrombin time for bull calves than for heifers; however, in analyzing the data with the t-test no significant difference statistically was found between sexes.

In Table III the average hematocrit values for 92 matured cows are given, average 35.08 ± 3.30%. There is an absence of breed effects but herd effects within the Jersey breed are significant at the 5% level. In calculating the significant differences among Jersey herds, a significant difference (5% level) was

TABLE III. Average Hematocrit Values of Matured Cows.

Breed	Herd No.	No. animals		Packed RBC (%)
Jersey	1	8		32.50 ± 1.41*
	2	10		35.05 ± 2.75
	3	18		35.50 ± 3.70
	4	22		35.45 ± 3.82
	5	8		36.13 ± 3.42
	Total	66	Avg	35.14 ± 3.48
Ayrshire	5	9		34.67 ± 2.50
Guernsey	5	8		36.00 ± 3.46
Holstein	5	9		34.22 ± 2.74
All breeds	Total	92	Avg	35.08 ± 3.30

* Stand. dev.

found between herds 1 and 3, 1 and 4, and 1 and 5.

Discussion. In Table I differences in plasma prothrombin time values among herds of Jersey breed and between Jersey and Guernsey breeds, that are of statistical importance, are probably influenced by rations. It has been shown in chickens(14) that feeding of terramycin lengthens blood clotting time. Other factors altering coagulation of blood are deficiency of vit. K(13), liver damage(13), poisonous plants(15,16), bacterial toxins(17), and halogenated hydrocarbons (18).

The plasma prothrombin time values for 10 animals fed trichloroethylene extracted soybeans(9) ranged from 21 to 28 sec., average 23.35. Averages reported in this paper are 23.20 on 115 matured cows and 24.82 on 90 calves. In no instance could the "incipient web of fibrin" be observed with blood plasma from cattle, as described by Quick (2) using human plasma, regardless of the number of variations placed upon the available light sources.

Numerous attempts were made to determine the plasma prothrombin time for calves immediately after parturition and before nursing. In all cases a clot did not form in the plasma with 0.02 M calcium chloride solution within 2 hours after mixing the contents of the test tubes. Sanford *et al.*(19) have shown that the % of prothrombin in blood plasma is slightly reduced in human infants. After birth the % of prothrombin is markedly reduced but the usual prothrombin time values are normally reached in 6 to 7 days. There is a similar trend in plasma prothrombin time of newborn calves. Greaves(20) and Bodansky(21) have attempted to explain the factors involved.

In some instances, it was found that a clot could not be obtained using a given plasma sample. Upon checking the time which had elapsed since the calcium chloride had been put into solution, it was found that 48 hours after preparation it was no longer effective in producing a clot in the mixture of plasma and thromboplastin. When a fresh solution of calcium chloride was prepared, a prothrombin time value well within the range of other samples that had been similarly processed was obtained. According to Guerrant(22) a change in the concentration of ionized calcium may have lengthened the prothrombin time. Wiggers(23) states that there is a maximum and minimum concentration of calcium chloride for the determination of plasma prothrombin time. When the concentration is not within this range, the prothrombin time is increased.

Summary. 1. The average plasma prothrombin time value of 115 matured dairy cows was 23.20 ± 4.42 and for 90 dairy calves 24.82 ± 4.47. The differences in the prothrombin time values among 6 herds of Jersey cows were highly significant (1% level) and significant (5% level) among breeds between Jerseys and Guernseys. The principal factor contributing to these effects was ascribed to rations. Age, breed, ration, and sex did not alter the prothrombin time values of dairy calves. 2. The holding of the blood plasma samples for 2.5 and 3.5 hours after collection of blood samples did not change the prothrombin time significantly when compared with the 0.5 hour values. 3. The plasma prothrombin time of newborn calves, prior to nursing, could not be determined since a clot or gel did not form in the blood plasma-thromboplastin mixture after the addition of calcium chloride. This finding corresponds with the observations made with blood plasma from human infants during the first week of life. 4. The average hematocrit value of 92 dairy cows consisting of Jersey, Guernsey, Ayrshire, and Holstein breeds was 35.08 ± 3.30. Breed differences in hematocrit values were not present; however, the differences among herds of the Jersey breed were significant at the 5% level.

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Hematogenous Pyelonephritis in Rats. II. Production of Chronic Pyelonephritis by *Escherichia coli.** (22157)

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Development of chronic pyelonephritis in man is obscure because usually the disease is examined only in its end stages. The present experiments were undertaken therefore to provide an experimental model for a controlled study of processes which culminate in chronic pyelonephritis. A major objective was to observe effects of chronic infection on the kidney in the absence of hydronephrosis or other structural abnormalities. We described previously a simple, non-surgical technic for producing acute hematogenous phylonephritis in rats, involving 2 steps; injection of *Escherichia coli* into blood, and massage of kidneys through intact body wall(1). The resulting disease was similar to acute pyelonephritis without manifest hydronephrosis in man, but self-limited, and subsided in approximately 6 weeks. To produce chronic pyelonephritis, rats from this study were subjected repeatedly for several months to bacterial injection and renal massage. Special interest was directed to the following problems: 1) relationship of chronic pyelonephritis to vascular disease and hypertension, 2) progression of chronic renal inflammation after cessation of active infection.

Materials and methods. Sixty-one male Sprague-Dawley rats weighing 150 to 200 g each, were divided into 5 groups of 12.† All were fed rat chow‡ and tap water *ad lib.* Treatments for each group are described in Table I. During the first 30 weeks, urine from rats in each group was obtained peri-

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† Group V consisted of 13 rats.

‡ Uncle Johnny Mills, Houston, Texas.

TABLE I. Schedule of Inoculations and Renal Massage.

Group	Time of inoculation of *E. coli* (wk)	Total No. of *E. coli* inoculated	Time of massage (wk)
I	0	10^8	Bilateral at 0, 9, 26
	7	10^5	
	9	”	
	26	10^8	
II	0	10^8	Unilateral (right side) at 0 and 9; bilateral* at 26
	7	10^5	
	9	”	
	26	10^8	
III	None		Bilateral at 0, 9, 26
IV	0	10^8	None
	7	10^5	
	9	”	
	26	10^8	
V	None		”

Inoculations made with 0.5 ml of 18 hr tryptose broth culture of *E. coli.* Strain used at 0, 7, and 9 wk was isolated from urine of patient with pyelonephritis and its virulence somewhat reduced by repeated subculture. The strain inoculated at 26 wk had been freshly isolated from blood of a second patient with pyelonephritis.

* Six of the 12 rats were given an additional inoculation of 10^8 *E. coli* and bilateral massage at 12 wk.

odically by cystotomy for culture and microscopic examination. Blood was removed by cardiac puncture at these times for culture and measurement of urea nitrogen. Blood pressure of each rat was determined every 2 weeks by tail plethysmographic method described by Williams, Grollman, and Harrison (2), with the rat lightly anesthetized with ether. Surviving animals were sacrificed after 42 weeks and at autopsy, studies of urine and blood were repeated. In addition, half of each kidney was ground to a pulp with sterile sand and cultured on blood agar, EMB agar, and in tryptose broth. The remaining half, as well as portions of heart, liver, spleen, and bladder were fixed in 10% formalin. Sections were stained with hematoxylin-eosin and Schiff's periodic acid.

Results. Serial studies before sacrifice. Twelve rats from Groups I and II failed to survive to 42 weeks; causes of death were anesthesia accidents in 5, bacteremic shock in 3, and unknown in 4. *E. coli* was isolated from urine at least once during life in all but 3 of the 12 rats which survived to 42 weeks

TABLE II. Summary of Bacteriologic and Pathologic Findings at Time of Sacrifice (42 Weeks).

Group‖	No. surviving rats	Positive urine cultures	Positive kidney cultures (No. of kidneys = 2 × No. of rats)	Positive blood cultures	BUN range and avg		Pyelonephritis†		Pyuria
					During life	At sacrifice	Gross	Microscopic	No. with pyuria/No. of rats
I & II—r.m. and inoc. of *E. coli*	12	1 *E. coli* 3 *P. inter.***	1 *E. coli*¶ 6 *P. inter.*	0	17-42 (28.6)	24-38 (29.7)	24/24	24/24	6/12§
III—r.m. only	10	0	2 *P. inter.**	1 *P. inter.*	25-33 (28.3)	26-34 (30.3)	0/20	1/20‡	0/10
IV—inoc. of *E. coli* without r.m.	6	0	0	0	20-33 (28.5)	Not done	1/12	3/12	1/ 6
V—controls; no inoc. or r.m.	13	0	4 *P. inter.**	2 *P. inter.*	17-40 (28.9)	24-27 (25.5)	0/26	1/26‡	0/13

* Isolation of *P. intermedium* probably resulted from blood in the kidney and not from renal tissues themselves since urines were negative and pyelonephritis was not present in these animals. † Numerator = No. of kidneys with pyelonephritis; denominator = total No. of kidneys examined. ‡ Questionable pyelonephritis only (see text). § Includes the 4 rats with positive cultures. ‖ r.m. = renal massage. ¶ One of the kidneys in this animal was sterile. ** *P. inter.* = *P. intermedium.*

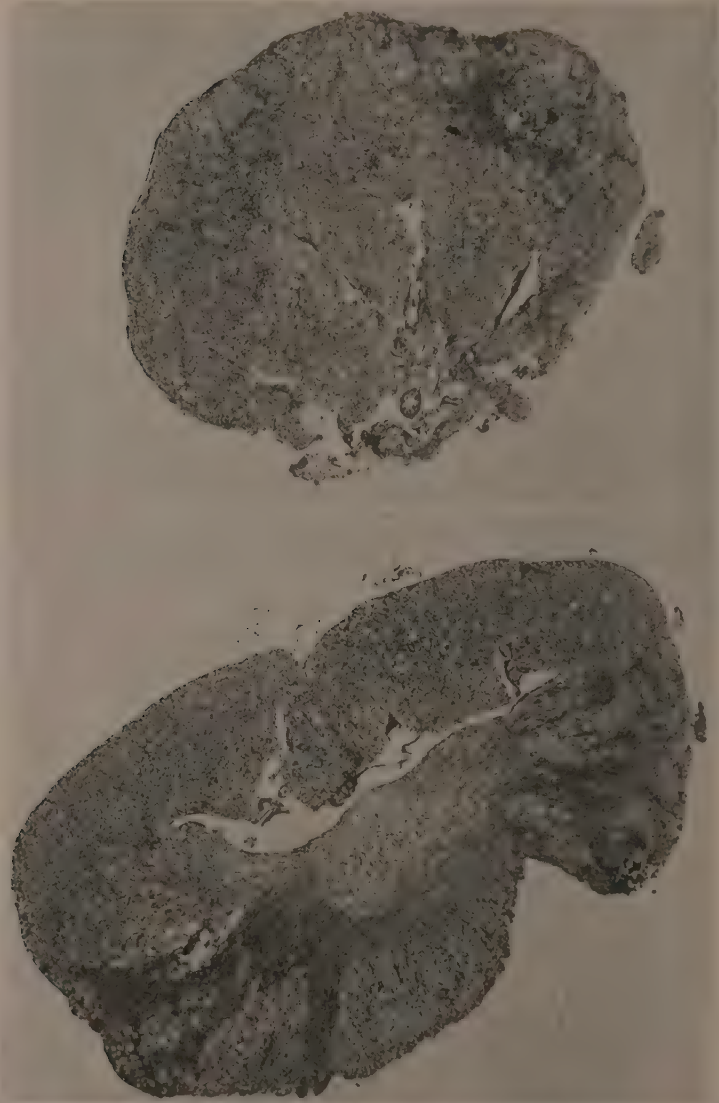

FIG. 1. Hilar cross sections of both kidneys from the same animal in Group I (× 4). Note marked differences in size, gross distortion produced by scarring, and loss of cortical substance. Urine culture had been positive for *E. coli* at 5 wk but both urine and kidney cultures were negative at 42 wk.

and in 6 dying earlier. Six rats in Group IV survived 42 weeks after the first inoculation of *E. coli*. Urines from 3 animals yielded positive cultures after first inoculation, but all subsequent urines from these and remainder of the group were negative. Two rats died of bacteremic shock and 2 died of unknown causes. Two were sacrificed at 36 weeks and negative cultures obtained from both urine and kidneys. In Group III, 2 deaths occurred from unknown causes; in Group V, all survived. All urine cultures in

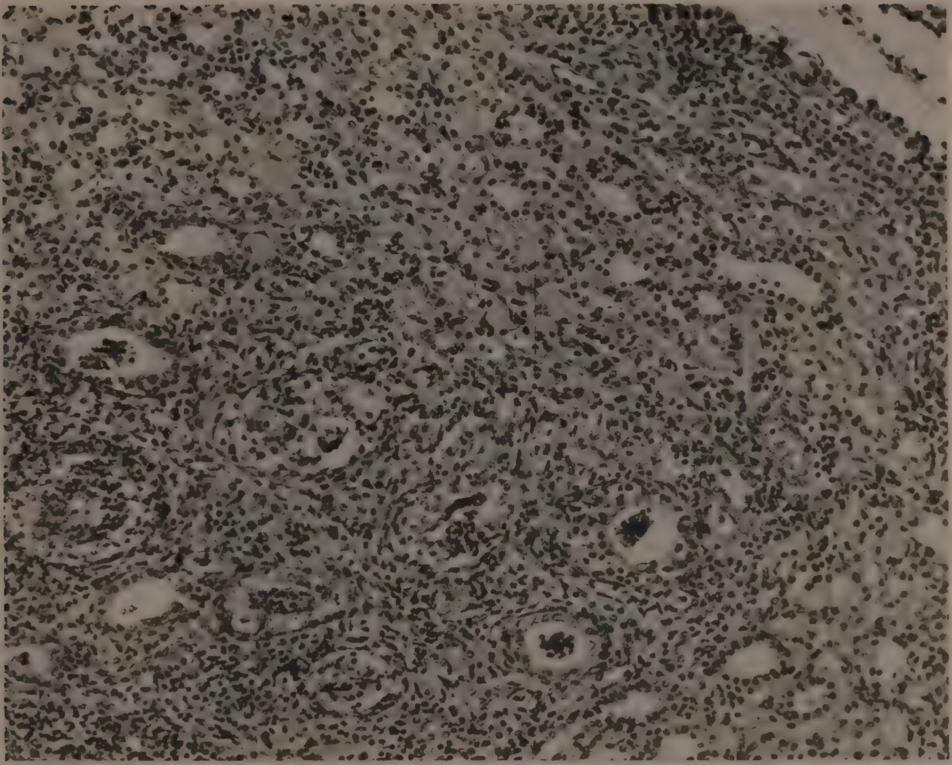

FIG. 2. Microscopic section of papillary tip of pyelonephritic kidney. Note fibrosis, mononuclear infiltration, and dilated tubules containing pus casts (×140). Urine cultures were positive for *E. coli* at 10 and 30 wk; *P. intermedium* was cultured from urine and kidneys at 42 wk.

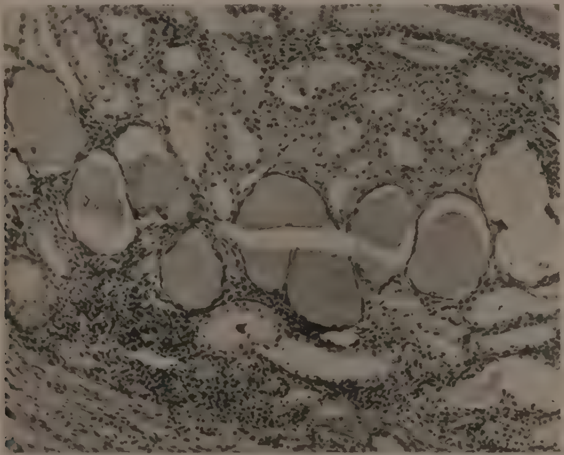

FIG. 3. Microscopic section from medullary area of pyelonephritic kidney revealing dilated, colloid-filled tubules. At the lower left, an area of fibrosis and mononuclear infiltration extends through medulla (×65). Urine culture positive for *E. coli* at 5 wk and negative at 15 and 42 wk; kidney culture negative at 42 wk.

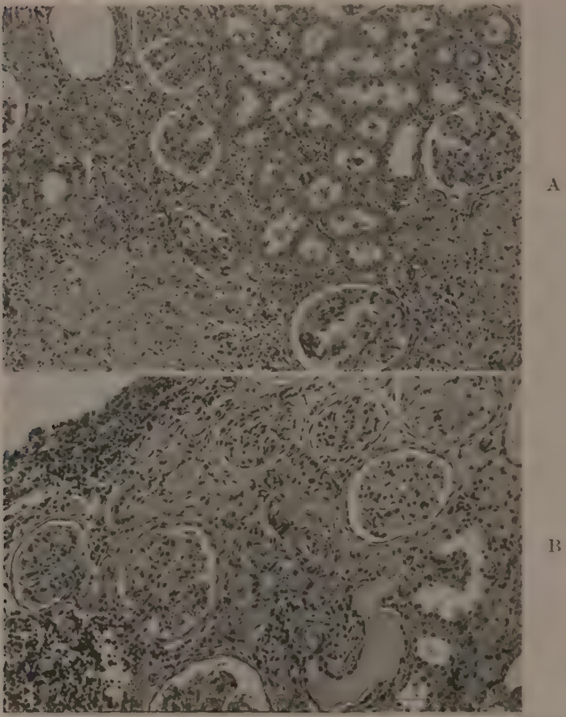

FIG. 4. Microscopic sections from 2 areas of cortex of pyelonephritic kidneys. A. Intact glomeruli with periglomerular fibrosis and marked interstitial fibrosis. Note interstitial accumulation of lymphocytes and plasma cells, and normal appearance of arterioles (×65). Cultures of urine and kidneys negative at 42 wk. B. Marked interstitial infiltration with mononuclear cells and widespread periglomerular fibrosis. Glomeruli themselves are normal (×70). Urine culture positive for *E. coli* at 5 wk, negative at 15 and 42 wk. Culture of kidney negative at 42 wk.

both groups were negative. Blood cultures repeatedly were negative in all five groups. Blood urea nitrogen was normal; values are listed in Table II.

Studies at completion of experiment. Bacteriological and pathological findings in all animals when sacrificed (42 weeks) are summarized in Table II. The 12 surviving animals in Groups I and II have been considered as a single group because all were subjected ultimately to bilateral renal massage. *E. coli* could still be isolated from only one rat in this combined group. From pyelonephritic kidneys and urine of 3 additional animals in the group, *Paracolobactrum intermedium* was cultured.

Pathologic findings. 1) Kidneys (Fig. 1-4). Changes of chronic pyelonephritis were found in all 12 rats in Groups I and II which survived 42 weeks (Table II). Four of the 12 animals dying earlier were examined, and 2 displayed pyelonephritic changes. The 2 in whom changes were absent had died after 12th week of the experiment. Kidneys were often adherent to surrounding organs and usually differed in size. They were distorted, lobulated by scars, and displayed loss of cortical substance and increased thickness of pelvic walls. Microscopic examination revealed areas of interstitial inflammation consisting of lymphocytes and plasma cells which extended throughout cortex and medulla. The tubules were dilated and many contained large colloid casts ("thyroid kidney"). Numerous "pus casts" were observed in kidneys from which positive cultures were obtained, but only a few in absence of demonstrable infection. There was extensive interstitial and periglomerular fibrosis but glomeruli themselves usually appeared normal. Significant vascular lesions were absent. Thickness of media of arterioles never exceeded that in controls. There was no intimal proliferation or medial necrosis. Rarely, an arteriole in the midst of a dense scar showed slight hyalinization. In 2 rats from Group IV, minimal findings of pyelonephritis were present, consisting of scattered infiltrations with lymphocytes and plasma cells and slight fibrosis; a single, small scar was observed grossly in one kidney. Among the remaining control animals (Groups III and V), occasional tiny depressions on surface of kidney were seen grossly, which corresponded to small areas of cellular infiltration beneath the capsule. A few tubules were dilated and contained colloid, but never to the degree seen in Groups I and II. In 2 rats with sterile kidneys, rare areas of cellular infiltration without fibrosis suggesting pyelonephritis were observed in cortex and medulla. 2) *Other organs.* Tiny well-healed scars were present in the hearts of many animals at site of needle puncture, but there was no other abnormality. Bladders in Groups I and II were often slightly thickened. Significant changes in spleen and liver were absent, except for splenic enlargement in animals with septicemia. *Blood pressure.* Mean systolic blood pressures and standard deviations of each group were: Group I and II: 111 ± 6; Group III: 116 ± 4; Group IV: 109 ± 5; Group V: 113 ± 6 mm Hg, respectively. Average blood pressures of pyelonephritic rats were thus not significantly higher than controls and hypertension was not observed in any individual animal, including those which died prior to completion of the study. Single determinations as high as 140 mm Hg[§] were noted rarely in animals from all groups but these were not sustained.

Discussion. Pathologic changes in kidneys of rats in Groups I and II closely resembled those seen in chronic pyelonephritis in man (3). The technic therefore provides an experimental model for examining problems related to the human disease.

The data suggest that recurrent, self-limited renal infections lead to permanent renal damage and inflammation which may persist even after infection has terminated. Although polymorphonuclear infiltration of kidneys and pyuria tended to diminish upon disappearance of active infection, an inflammatory reaction remained, characterized by infiltration of plasma cells and lymphocytes and accompanied by proliferation of connective tissue. Studies are presently underway to determine whether these sterile inflammatory lesions gradually subside or progress in the absence of reinfection and lead to renal failure.

Bacteremia due to *Paracolobactrum intermedium* noted in 3 control animals failed to produce pyelonephritis or infected urine. In 3 pyelonephritic rats in Groups I and II, however, *P. intermedium* was associated with pyuria and infected urine. These data suggest that the diseased kidney is rendered more vulnerable to infection by secondary invaders.

In contrast to the impression gained by investigators from clinical-pathologic correlations in the human(3), there was no evidence in these animals that hypertensive vascular disease develops as a direct sequel to the inflammatory process. Although extensive pathological damage was produced in these rats, areas of normal kidney were present and sufficient renal function remained to prevent azotemia. Observations over longer periods should help to determine whether more severe impairment of renal function must develop or some other factor affecting arterioles must be imposed before vascular disease appears.

Summary and conclusions. 1) Chronic bilateral pyelonephritis without manifest hydronephrosis was produced experimentally in the albino rat by a simple non-surgical technique. This experimental disease provides a model for study of human pyelonephritis. 2)

[§] The average systolic blood pressure of normal Sprague-Dawley rats as determined with the tail plethysmographic method in this laboratory is 110 mm Hg with a standard deviation of ± 10. A rise of 5 to 10 mm Hg occurs with age. Rats in whom experimental hypertension is produced by renal ischemia reveal blood pressures of 150 mm Hg and above, with this method.

Renal inflammation tended to persist after infection had disappeared. 3) Vascular disease and hypertension did not develop; however, the lesions were of insufficient severity to produce azotemia.

We are indebted to Dr. Leo Weiss, Chief of the Pathology Service, Dallas Veterans Administration Hospital, for his assistance in interpretation of microscopic findings.

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Purification of Hyaluronidase from *Clostridium perfringens.** (22158)

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Although several investigators(1-4) have prepared highly purified hyaluronidase from bovine testicular tissue, bacterial hyaluronidases have not usually been purified to a comparable degree. Most authors(5) have used either simple filtrates of the bacteria or crude $(NH_4)_2SO_4$ precipitates. The notable exception was the discovery by Rogers(6) that crude precipitates from ammonium sulphate treatment of filtrates of *Clostridium perfringens,* Type A, Strain 107, could be absorbed on aluminum oxide at pH 4.6 and eluted at pH 7.0 to give a 16-fold increase in potency of hyaluronidase preparations. This method is difficult to reproduce and Rogers was not always able to get a sharp separation with his preparations. For this reason we initiated an alternate method for purification of the bacterial hyaluronidases. This report deals with the preparation of highly purified hyaluronidase from *Cl. perfringens, culture 2278.*

Methods. The hyaluronidase unit was based on the unit of testicular hyaluronidase previously reported(7). Due to instability of the bacterial filtrates at pH 5.5, the enzyme was incubated with hyaluronic acid at pH 7.0. Fig. 1 shows that turbidimetric curves coincided in the range of 2 to 5 units. Fractions for assay were diluted with a buffered solution of 0.1% bovine serum albumin(8) until they fell within these limits. Nitrogen content of various fractions was determined by micro-Kjeldahl digestion with selenium-sulfuric acid followed by nesslerization. Three strains of *Clostridium perfringens* were studied. A lyophilized stock strain, #47-E-2, Walter Reed Army Institute of Research collection, represented the so-called laboratory strains. It had been held in dried state since 1944, but there were no records of its source. A second culture, #4209, isolated in 1951,

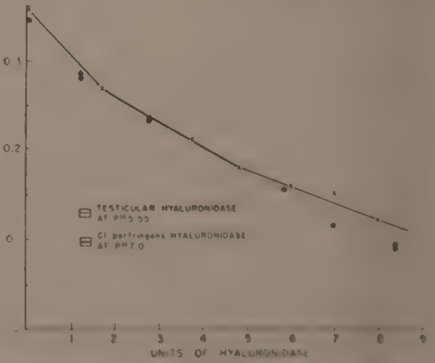

FIG. 1. Comparison of relationship of optical density to *Cl. perfringens* hyaluronidase and testicular hyaluronidase.

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[*] Presented in part before the Soc. of Am. Bact., Pittsburgh, Pa., May 1954.

[†] Abstracted in part from thesis presented to Graduate School, Georgetown University, in partial fulfillment of requirements for the degree of Doctor of Philosophy.

from a gangrenous war wound represented a strain of known pathogenicity. It was isolated and lyophilized at the 406th Medical General Laboratory (Tokyo) approximately one year prior to these studies. A third strain, #2278, had been isolated from the gangrenous wound of a patient at Walter Reed Army Hospital in 1952. This culture had been maintained on blood agar plates in an anaerobic atmosphere and transferred at monthly intervals.

Results. Choice of strain and medium. Considering the nutritional selectivity of *Clostridia* 3 types of culture media, at pH 7.6, were studied to obtain the greatest amount of bacterial filtrate: Difco brain heart infusion broth containing 1.5% yeast extract, Bernheimer's synthetic medium, and trypticase lactose iron broth. Starter cultures for mass inoculation were prepared by seeding 1 liter of each of these media with 10 ml of thioglycollate broth from the blood agar plates on which the various strains had been initially recovered from stock. Good growth was obtained after 16 hours incubation at 35°C. One liter quantities of the 3 selective media were prepared in the usual manner. Excess oxygen was displaced by boiling and cooling to approximately 40°C immediately prior to adding 10 ml of thioglycollate broth starter culture. The 1 liter flasks were next sealed with parafilm and incubated at 35°C for 18 hours, after which the cultures were centrifuged. Supernatants were adjusted to pH 9.0 with 1N sodium hydroxide and sterilized by Seitz filtration.

Hyaluronidase yields by strains of *Clostridia* employed were widely divergent. All strains failed to grow in Bernheimer's synthetic medium. Fair growth was obtained in trypticase lactose iron broth, but hyaluronidase yield was not satisfactory. The failure of both media to support good growth of *Clostridium perfringens* was unexpected. Best results were obtained in brain heart infusion broth with 1.5% yeast extract added. The hyaluronidase yield in this medium was 10 times that obtained in trypticase lactose iron broth and gave values of 0, 60 and 100 units/ml of culture filtrate for strains #47-E-2, #4209 and #2278 respectively. Within the small number of strains tested, there appeared to be a correlation between length of time these cultures have been held in the dried state and their ability to produce hyaluronidase. Culture #47-E-2, in the dried state for more than 10 years, failed to produce the enzyme, although it grew well. Culture #4209, lyophilized in 1951, produced small amounts of hyaluronidase. On the other hand, culture #2278, which had never been lyophilized, produced by far the most enzyme. This culture was used for subsequent preparations of purified hyaluronidase.

Preparation of purified hyaluronidase. Preliminary attempts to fractionate the bacterial filtrate at pH 7.6 were completely unsuccessful. The enzyme appeared to be stable in the filtrate at this pH for only 72 hours and then was rapidly destroyed. Precipitation with 0.7 ammonium sulphate or with ethanol appeared to hasten destruction of the enzyme. Stabilization of the original culture filtrates, and the first crude fractions, was necessary. Since instability of the hyaluronidase might be due to proteolysis by other enzymes in the culture filtrates, the effect of pH on stability of the hyaluronidase at 0°C was investigated, using the culture filtrates and the 0.7 saturated ammonium sulphate precipitate buffered to several pH values with and without ethanol at 0.3 mole fractions. The 0.3 mole fraction of ethanol was selected to represent maximum concentration of ethanol anticipated in subsequent experiments. Below pH 7.0 destruction of the enzymes was exceedingly rapid. At pH 7.0 in the buffer, the enzyme showed a minor loss in 1 hour but was completely destroyed in 24 hours. In ethanol it was completely lost. Between pH 8.6 and 9.5 the enzyme remained indefinitely stable in either buffer or buffer containing ethanol. At lower hydrogen ion concentrations only the buffered enzyme remained stable. Consequently, the cultures of *Cl. perfringens* were immediately adjusted to pH 9.0 after the bulk of organisms had been removed by centrifugation at 4000 rpm and supernatant clarified by

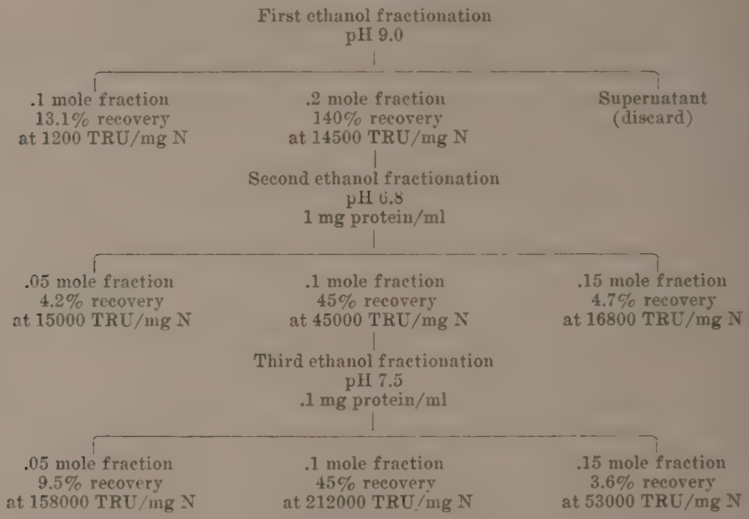
First ethanol fractionation
pH 9.0
.1 mole fraction
13.1% recovery
at 1200 TRU/mg N
.2 mole fraction
140% recovery
at 14500 TRU/mg N
Supernatant
(discard)
Second ethanol fractionation
pH 6.8
1 mg protein/ml
.05 mole fraction
4.2% recovery
at 15000 TRU/mg N
.1 mole fraction
45% recovery
at 45000 TRU/mg N
.15 mole fraction
4.7% recovery
at 16800 TRU/mg N
Third ethanol fractionation
pH 7.5
.1 mg protein/ml
.05 mole fraction
9.5% recovery
at 158000 TRU/mg N
.1 mole fraction
45% recovery
at 212000 TRU/mg N
.15 mole fraction
3.6% recovery
at 53000 TRU/mg N

FIG. 2. Ethanol fractionation of hyaluronidase from *Cl. perfringens.*

Seitz filtration. Solid ammonium sulphate was added to the clear filtrate to 0.3 saturation. The suspension was allowed to stand 24 hours at 4°C. The inactive precipitate was removed by centrifugation and discarded. Ammonium sulphate was added to the supernate to 0.7 saturation, and the suspension was again allowed to equilibrate for 24 hours at 4°C. The active precipitate was removed by centrifugation and dissolved in one-fifth the original volume with 0.02 molar veronal buffer, pH 9.0, containing 0.45% NaCl.

The ethanol fractionations were conducted as previously described(2) except that suspensions were allowed to equilibrate for 24 hours after addition of each increment of ethanol and precipitates removed in refrigerated centrifuge, –5°C, at 4000 rpm. Application of this technic to testicular hyaluronidase yielded 15 to 25% recovery of the crude enzyme and a purified preparation assaying 160000 TRU/mg N. Typical results of the first ethanol fractionation are shown in Fig. 2. The recovery figures are based on the hyaluronidase content of 3.5 l of culture filtrate which contained 100 TRU/ml at an index of purification of 200 TRU/mg N. The ammonium sulphate precipitation in combination with the first ethanol fractionation therefore caused a 70-fold purification of the bacterial hyaluronidase. Recovery in the dialyzed 0.1 and 0.2 mole fractions of 153% of the total enzyme, can be partly explained by the increased stability of these fractions at pH 7.0 where the assay was conducted. The apparent anomaly may also be due to removal of inhibitors present in the original culture filtrate. As this stage the 0.2 mole fraction was stable indefinitely at pH 7.3 in buffered solution and was stable for at least 72 hours at pH 7.0.

Previous work suggested that the greatest purification could be achieved by fractionating next at a low pH. Consequently, pH 6.8 was chosen for the second ethanol precipitation. The 0.2 mole fraction from the first ethanol was dialyzed, and concentrated phosphate buffer containing NaCl was added to 0.02 M phosphate, pH 7.3 containing 0.45% NaCl. The solution was diluted with buffer to 1 mg protein/ml, and the pH adjusted to 6.8. Fig. 2 shows destruction of the enzyme at this pH since only 54% was recovered. However, the 0.1 mole fraction contained 45% of the original enzyme 3 times purer.

The third ethanol fractionation was conducted at pH 7.5. The 0.1 mole fraction from the second ethanol treatment was dissolved in the 0.02 M phosphate buffer pH 7.3, diluted to 0.1 mg protein/ml and pH was adjusted to 7.5. It is believed that the 4-5 fold increase in purification shown in Fig. 2, is due more to the very dilute solution of protein fractionated rather than change in pH. As in the first ethanol fractionation over 100% recovery of the enzyme being fractionated was obtained. This increase could not be attributed to increased stability of the enzyme at assay pH of 7.0, because the enzyme was stable at this pH for at least 3 weeks after the second ethanol fractionation. It seems more likely to be due to removal of inhibitors during the purification procedure.

An apparent over-all recovery of 45% was obtained by this procedure and the enzyme was purified 1000-fold. However, it is obvious from the data that removal of inhibitors during purification may account for part of this high yield. In small scale experiments, it appeared that if the second ethanol was conducted at pH 7.0 instead of 6.8 even higher yields could be obtained.

Rogers(6,9) does not mention the loss of *Cl. perfringens* hyaluronidase below pH 7.0. In fact, we may assume that this did not occur with his strain since it was absorbed on aluminum oxide at pH 4.6 (a pH which would have caused immediate destruction of hyaluronidase preparations obtained in these laboratories). The marked difference in stability of these two hyaluronidase preparations cannot be explained at present. Conditions of growth, as well as strains, permit many possibilities. However, it is suggested that two hyaluronidase disaccharases may be present in these organisms. Consequently, the two laboratories may not have been dealing with the same enzyme.

Summary. A procedure has been detailed for a 1000-fold purification of a bacterial hyaluronidase from *Cl. perfringens,* strain 2278. Initial crude fractionation with ammonium sulphate followed by three ethanol fractionations at pH 9.0, 6.8 and 7.5 respectively have yielded hyaluronidase fractions containing 200000 TRU/mg N with recovery of 45% of the crude enzyme.

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Production of Impending Hepatic Coma by a Carbonic Anhydrase Inhibitor, Diamox.*† (22159)

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Impending hepatic coma, characterized by confusion and the typical "flapping" tremor, has been described previously in alcoholics with cirrhosis given oral nitrogenous substances—ammonium cation exchange resins, ammonium salts, urea, and increments in dietary protein(1-3). The onset of this syndrome is here described in similar patients following oral administration of a carbonic anhydrase

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[†] A preliminary report is included in the *Proc. Am. Soc. Clin. Investig.,* May 1955.

TABLE I. Clinical and Laboratory Data of 12 Alcoholics with Cirrhosis Given an Oral Carbonic Anhydrase Inhibitor, Diamox.

Patient, age, sex	BSP†/Ser. bil. (mg/100 ml)	Diamox–Dose, frequency,‡ duration	KCl daily dose (g)	Mental state during Diamox‡	"Flapping" tremor during Diamox	Avg periph. vein blood NH_3-N** (γ/100 ml) Before§	During‖
		Group I (Developed impending coma during Diamox)					
		mg Days					
J.H.,* 49, ♀	35/ 1.2	500 A 3	0	C	Appeared	—	—
		" A 13	6	C	"	51 (2)	138 (8)
		" D 6	6	C	"	81 (2)	105 (4)
			Started 3rd day				
J.M.,*¶ 32, ♂	36/ 4.5	" A 3	0	C	"	—	—
J.S.,¶ 44, ♂	—/ 8.0	" D 7	3	C	More severe	65 (3)	116 (4)
J.T.,¶ 50, ♂	33/ 3.5	1000 D 5	0	C	0	55 (3)	50 (3)
		Group II (No impending coma)					
H.D.,* 40, ♀	48/10	500 A 27	0	Dr	0	63 (4)	87 (17)
	16/ 1.2	" D 3	0	N	0	78 (1)	69 (1)
E.F.,* 37, ♀	—/ 4.3	" A 27	0	N	0	62 (3)	87 (19)
J.Mc.,* 45, ♂	25/ 1.3	" D 7	0	N	0	74 (2)	89 (5)
G.A., 52, ♂	—/15.6	" D 6	0	N	0	85 (4)	92 (4)
F.J., 62, ♂	31/ 2.0	" D 8	0	N	0	59 (3)	66 (4)
C.O'D., 49, ♀	29/	500 D 7 Then 1000 D 2	0	Dr	0	73 (4)	88 (6)
G.D., 42, ♀	51/ 4.7	1000 D 7	0	N	0	64 (3)	73 (4)
M.B., 47, ♀	—/ 6.9	" D 5	0	Dr	0	48 (2)	55 (4)

* Diagnosis of Laennec's cirrhosis confirmed by postmortem or liver biopsy.
† Bromsulfalein: % retention in 45 min.
‡ A = Alternate days; D = Daily; C = Confused; Dr = Drowsy; N = Normal.
§ Before Diamox administration.
‖ During " " .
¶ History of previous mental symptoms associated with liver disease.
** Numbers in parentheses represent the number of determinations.

inhibitor, acetazolamide (Diamox)[‡](4,5). This drug, as well as sulfanilamide, has been employed in the treatment of cardiac failure because it may cause a sodium and water diuresis(6,7).

Materials and methods. From January, 1954, through January, 1955, on the Thorndike Ward, 12 alcoholic patients with cirrhosis received 15 courses of Diamox therapy (Table I). One patient received 3 courses, another 2. Six patients were men and 6 women, ranging from 32 to 62 years of age. Diagnosis of cirrhosis was made by history of prolonged alcohol and poor food intake coupled with physical and laboratory evidence consistent with severe long-standing liver disease. Six of the 12 patients had mild hemolytic anemias thought to be consistent with liver disease(8) and 3 had peripheral neuritis. Rheumatic heart disease, treated "beri-beri" heart disease, and psoriasis were diagnosed in one instance each. Diagnosis of cirrhosis was confirmed by postmortem examination in patients J. M. and J. H., who died several months later, and by liver biopsy in 3 other patients. One of the latter, J. Mc., also had peritoneal tuberculosis when biopsy was performed 3 months after Diamox. All patients had fluid accumulation and received weighed diets containing less than 270 mg of sodium and from 66 to 75 g of protein daily. An oral vitamin supplement· Vi Magna Granules,[§] containing vit. A, B complex, C, and D, was added daily. Stools were examined frequently for occult blood. Blood was drawn in the fasting state or 2 to 3 hours postprandially in the morning. Hematocrits, serum carbon dioxide contents, and concentrations of nonprotein nitrogen, sodium, potassium, and chloride, were determined using methods previously described(9). Serum bilirubin, 45-minute bromsulphalein retention, cephalin flocculation, thymol turbidity and flocculation were also determined using standard methods. One ml of peripheral venous blood drawn in a heparinized syringe was immediately injected into the alkaline outer chambers of each of 2 Conway units and analyzed for "ammonia" nitrogen (NH_3-N)[‖] using McDermott and Adams' modification of the Conway technic(10). Our values were lower than those of McDermott *et al.*, but recoveries from standard solutions were good. The normal range was from 29 to 86 mg NH_3-N per 100 ml (16 patients, 19 determinations). Before Diamox 71 control determinations in 12 patients with cirrhosis ranged from 28 to 114 μg per 100 ml with only 2 determinations exceeding 100. Eight patients with spontaneous impending hepatic coma, not included here, had concentrations (21 determinations) ranging from 35 to 246 μg per 100 ml and 6 of these were below 114. Blood NH_3-N determinations during Diamox administration were made on the morning following initial dose and most mornings thereafter up to and including the morning the drug was discontinued. Concentrations presented (Table) represent averages; figures in parentheses represent the number of determinations from which averages were calculated. After blood NH_3-N determinations were made, Diamox was given in amounts of from 500 mg on alternate days to 1000 mg daily from 3 to 27 days. Potassium chloride was given in daily oral quantities of 3 g to one patient (J.S.) and 6 g to another (J.H.); the latter received it before and during one course of Diamox and also during part of another (Table).

[‡] Provided through the courtesy of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[§] Provided through the courtesy of J. M. Ruegseggar of Lederle Laboratories Division of American Cyanamid Co., Pearl River, N. Y.

Confusion and the typical "flapping" tremor(11) were not present before Diamox except in J. S., who had a slight tremor.

Results. Two groups were established (Table): Group I, 4 patients who developed confusion with or without tremor, and Group II, the remaining 8, none of whom developed this syndrome. All in Group I had chronic severe cirrhosis and 3 had previously had impending hepatic coma. Of the 8 in Group II, 5 had cirrhosis of a severity comparable to those in Group I, but none had had impending coma.

Beginning within 2 to 48 hours of the initial dose, 4 patients developed confusion, during 6 periods of Diamox administration, 2 after only 500 mg. Tremor remained absent during one period, became accentuated in one, and appeared in 4. Confusion became marked in 2 patients (J.H. and J.M.) and remained mild in the other 2. Except in J.M. it did not progress after the first 48 hours and improved in J.S. and J.T. despite continuation of Diamox. The tremor usually persisted longer.

During Diamox administration average venous blood NH_3-N concentrations rose above those of the highest control determination (114 μg per 100 ml) during 2 of 4 periods. During Diamox, average NH_3-N concentrations and increases in these 2 instances exceeded all control averages in Group II at the 5% level or less. Concentrations fluctuated from day to day and were usually higher in the first 72 hours, falling later despite continuation of Diamox. Average serum potassium concentrations decreased during all 6 periods, during 4, 0.4 mEq or more. Potassium chloride, administered during 3 periods, failed to prevent or alleviate impending coma although average serum potassium concentrations remained above 4 mEq per l in patients who received it.

In Group II, confusion and tremor were absent, although drowsiness was present in 3 of 8 patients during Diamox administration. Average blood NH_3-N concentrations increased during all except one period. Day to day values, highest in the first 72 hours, fluctuated, sometimes reaching abnormally high levels. In one of 7 periods the average serum potassium concentration decreased more than 0.4 mEq per l, and during another a low value (2.7 mEq per ml) was found.

[‖] Ammonium ion is either present in shed blood or formed from nitrogenous substances in blood shortly after shedding. Volatile blood base, liberated by adding strong alkali, is probably all or nearly all ammonia.

The 2 groups showed no significant difference in serum carbon dioxide contents or concentrations of sodium and chloride before or after Diamox. Serum sodium concentrations rose slightly during 6 and remained constant during 8 periods of Diamox therapy; serum chloride concentrations rose during 9 periods, remained the same during 3, and dropped slightly during one. Serum carbon dioxide contents decreased during 11 periods and remained the same in 3. Acidosis (a rise in serum chloride concentration and fall in carbon dioxide content) occurred even when the smallest doses of Diamox were given.

Discussion. Clinical signs and symptoms which may occur after Diamox administration are well documented by Maren *et al.*(12). Noted in patients without liver disease have been paresthesias, drowsiness, and deep sleep. Confusion was not reported and we did not observe it in 12 patients given single 500 mg oral doses of Diamox.

In cirrhosis of the liver, however, bizarre changes of mood, hallucinations, loss of memory, confusion, and occasional psychotic episodes were first described by Eisenmenger (13) to occur following Diamox administration. Confusion also appeared here, but was, in addition, often associated with the typical "flapping" tremor which occurs in impending hepatic coma.

Impending hepatic coma, characterized by confusion and "flapping" tremor in patients with liver disease, is often but not always associated with increased venous blood NH_3-N concentrations(14). Two of our 3 patients with confusion and tremor had significant increases and elevated concentrations of blood NH_3-N after Diamox. The nitrogen contained in Diamox was not the source of increased blood NH_3-N because in cirrhosis this drug is nearly all excreted in the urine(13). The pathogenesis of impending hepatic coma after Diamox is different, then, from that induced by nitrogenous substances thought to liberate ammonium in the gastrointestinal tract.

Diamox may have a direct effect on brain metabolism because parasthesias occurring in the first 8 hours after Diamox improved as the plasma concentrations of Diamox could be predicted to be falling and because administration of sulfanilamide, another carbonic anhydrase inhibitor, produced confusion in a large percentage of patients without liver disease(12). The mechanism of action is unknown, although sulfanilamide interferes with maintenance of potassium gradients in guinea pig brain slices(15). Decreases in serum potassium concentrations were more marked here in the group developing impending coma, but mental symptoms occurred at normal serum potassium concentrations and potassium salts produced no improvement. Possibly, the brain of patients with severe hepatic disease is more sensitive to Diamox because only these patients developed confusion in our hands.

Conclusions. 1. The carbonic anhydrase inhibitor, Diamox, can produce impending hepatic coma in susceptible alcoholics with cirrhosis. 2. This type of induced impending coma may be associated with a rise in femoral or antecubital vein blood ammonia nitrogen concentrations to levels above those seen in mentally clear patients with cirrhosis. 3. The nitrogen contained in Diamox is not a source of the elevated blood ammonia nitrogen concentrations.

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Residual Organ Blood Volume of Cattle, Sheep and Swine.* (22160)

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In quantitative studies involving investigations of tissue distribution of blood-borne substances a correction for the residual organ blood may be essential. This is of special importance in short time radioisotope distribution experiments where retained tissue blood may be sufficient to contribute significantly to the total activity recovered. Specific studies based upon whole tissue extractions measure not only the substance in the cells of the tissues, but also that existing in the blood which is trapped within these tissues at the time of sampling. Therefore, in tracing the distribution of an ingested or injected substance by extraction from various organs, it is necessary to know the proportion of the material that is from the tissue and that which is from the blood itself. When the relative proportions of blood and tissue are known, precise quantitative studies on movement and tissue composition may be used to substantiate microscopic examinations and organ weight changes. The total organ weights for farm animals have been reported by several investigators(1-3), and procedures and values for blood volume of cattle, sheep and swine are in the literature(4-6). Lewis *et al.*(7) have reviewed procedures and reported the total organ blood volume measurements for the rat. Other investigators have made *in vitro* and *in vivo* measurements of total organ blood volumes in laboratory animals(8,9) but there were no data available from the literature directly related to the residual blood present in organs and tissues of farm animals slaughtered by the conventional procedures.

This report is concerned with the residual blood values as related to age and species for the various organs and tissues of farm animals routinely sacrificed and bled out during current nutritional investigations.

Material and methods. The cattle, sheep and swine used in these studies were normal, healthy individual animals maintained under general farm management conditions. Total red blood cell volumes were obtained by the injection of P^{32} labeled homologous erythrocytes as previously described(4). The organ blood values were procured as follows: (a) blood samples were drawn from the animal; (b) whole blood was incubated with P^{32}; (c) red blood cells were washed sufficiently to remove any radioactivity not in the cells and (d) the cells were reconstituted to the original volume with the animal's own plasma. (e) A known volume of suspended labeled cells was re-injected into the immobilized animal(10) and after mixing had occurred, blood samples were taken from the jugular vein of the animal, usually at 5 and at 15 minute intervals for radioactivity measurement and blood volume determinations. (f) The animal was then sacrificed by a blow

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TABLE I. Mean Organ Weights for Cattle, Swine and Sheep.

Species	Age (mo)	No. of animals	Mean wt (lb)	Fresh wt as % of body wt ± stand. dev.					
				Blood*	Liver	Kidney†	Spleen	Lungs	Heart
Cattle	.5 3	12	88	8.4	2.31 ± .71	.230±.035	.359±.106	1.57 ±.42	.767±.242
	6	15	384	6.0	2.24 ± .19	.124±.024	.266±.021	.821±.12	.457±.087
	Aged	8	923	5.6	2.17 ± .06	.130±.032	.256±.024	.859±.120	.471±.051
Sheep	.5	5	10	9.3	1.69 ± .24	.560±.30	.320±.28	1.65 ±.05	.740±.01
	4	6	80	6.5	1.84 ± .24	.140±.02	.240±.03	1.08 ±.10	.360±.12
	Yearlings	8	118	5.9	1.30 ± .29	.120±.02	.260±.02	1.00 ±.30	.390±.08
Swine	.5	7	10	7.2	2.52 ± .25	.415±.046	.297±.125	1.29 ±.22	.609±.163
	2	11	35	6.7	2.10 ± .26	.299±.048	.220±.066	1.21 ±.22	.472±.091
	5	6	102	6.3	2.66 ± .34	.242±.061	.232±.051	.95 ±.20	.403±.101
	Aged	6	654	3.5	1.859± .22	.820±.0228	.218±.029	.834±.117	.319±.039

* ml of blood/100 g body wt. † Mean wt of one kidney only.

on the head, hoisted by the rear legs and bled out for 5 minutes. The organs and tissues were immediately excised, blotted free of excess blood and weighed, (g) whole organs or 10 to 15 samples were taken from each organ or tissue being studied for the measurement of radioactivity, and (h) these average values were used for the estimation of the residual blood present. All tissue samples were weighed and wet ashed with nitric acid, made to volume and aliquots taken for radioactivity measurements by conventional procedures as described by Hansard *et al.*(4). The milliliters of blood per 100 g of tissue were then calculated from the isotope concentration in the samples and the blood volume standard (5).

Results. Organ weights of farm animals. The mean unit weights of the various organs of cattle, sheep and swine employed are listed in Table I as a percentage of the live body weight. These average values compare favorably with those reported by other investigators(1-3). It is noted that there was more difference due to weight of animal than to species. The very young of all species showed a higher blood volume and slightly higher mean organ weights. These values are included to provide a means of estimating the total residual blood present in whole organs and tissues.

Residual blood in cattle organs and tissue. The residual blood in organs of 7 cattle in 2 age groups is shown in Table II. There was a wide variation between individual tissues but the total blood volume values were similar to those reported previously(4). In these animals the spleen retained the highest concentration of residual blood, followed by the lungs, liver, kidney, pituitary, adrenal, heart, pancreas and muscle in that order. Age appeared to affect concentration of retained

TABLE II. Residual Organ Blood Volume of Cattle at Various Weights.

Wt (lb)	Total blood vol (ml 100 g)	ml blood/100 g (fresh wt)									
		Spleen	Lungs	Liver	Kidney	Pituitary	Adrenal	Heart	Pancreas	Loin muscle	G. muscle
70	7.6	31.6	23.2	10.4	3.9	2.8	3.1	2.9	2.3	1.3	1.1
78	9.1	29.7	26.2	6.1	3.4	4.0	2.8	2.2	2.2	1.2	.8
83	8.5	32.6	25.8	7.2	3.7	3.4	3.2	2.7	3.1	1.2	1.0
Avg 77 ± 7.7*	8.4 ± .9	31.3 ±1.7	25.1 ±1.8	7.9 ±2.5	3.7 ±.3	3.4 ±.7	3.0 ±.2	2.6 ±.4	2.5 ±.5	1.2 ±.06	.96 ±.12
800	5.6	22.1	23.4	9.5	7.0	4.6	3.9	4.4	4.5	.7	.7
758	5.8	39.3	18.2	6.0	6.9	4.3	3.5	3.0	3.2	.9	1.1
834	5.7	39.4	18.2	7.3	5.9	3.7	3.8	3.4	2.8	1.2	1.0
992	5.4	37.0	21.5	8.0	6.6	5.8	4.1	3.9	2.9	1.3	1.1
Avg 846 ± 115	5.6 ±.2.	34.5 ±3.6	20.3 ±2.5	7.7 ±1.7	6.6 ±.5	4.6 ± 1.	3.8 ±.3	3.7 ±.7	3.3 ±.8	1.0 ±.3	1.0 ±.2

* Mean ± S.D.

TABLE III. Residual Organ Blood Volume of Sheep at Various Weights.

Wt (lb)	Total blood vol (ml/100 g)	ml blood/100 g (fresh wt)									
		Spleen	Lungs	Liver	Kidney	Heart	Pancreas	Adrenal	Pituitary	Loin muscle	G. muscle
6	10.0	33.0	16.3	10.3	6.2	5.3	4.0	1.7	1.9	2.6	2.0
10	9.3	36.1	18.2	9.8	5.7	6.3	3.2	1.9	2.2	1.3	1.4
12	8.5	35.4	20.1	8.6	4.8	6.2	2.1	2.1	2.0	1.8	1.6
Avg 9	9.3	34.8	18.2	9.6	5.6	5.9	3.1	1.9	2.0	1.9	1.7
± 3.5*	± .9	±1.8	± 2.2	± 1.	± .8	± .6	± 1.1	± .2	± .2	± .8	± .4
96	6.2	33.0	29.4	9.1	5.3	5.5	4.0	2.2	2.1	1.1	1.5
102	6.1	36.8	29.0	6.6	5.3	4.4	2.5	4.2	2.0	.9	.7
136	5.5	34.2	21.5	8.6	6.4	5.5	3.3	3.1	2.5	1.5	1.2
Avg 111	5.9	34.7	26.6	8.1	5.4	5.1	3.3	3.2	2.2	1.2	1.1
± 24	± .4	±2.2	± 4.7	± 1.5	± .6	± .6	± .9	± 1.2	± .3	± .4	± .5

* Mean ± S.D.

blood, but the differences between old and young cattle were not consistent nor sufficient for significance in this study.

Residual blood in sheep organs and tissue. The milliliters of blood per 100 g of fresh tissue are shown for old and young sheep in Table III. The trend followed essentially the same pattern as was indicated for cattle, and the milliliter percentage of residual blood closely paralleled that for organs and tissues of cattle. When consideration is given to the fact that one-third and one-fourth of the weight of the spleen and of the lungs respectively were due to blood itself, the significance of this dilution factor for the interpretation of tissue analysis values can be realized. Other tissue and organ blood values ranged from one to 10% on a fresh weight basis.

Residual blood in swine organs and tissue. Residual blood determinations were made in tissues and organs from 10 swine at different ages and weights as indicated in Table IV. The calculated milliliters of residual blood per 100 g of fresh tissue show the lungs to be highest, followed by the spleen, kidney, liver, adrenals, heart, pancreas, loin muscle and gastrocnemius muscle in that order. There appeared to be a general tendency toward a higher concentration of residual blood in organs and muscle of the younger animals.

Discussion. The isotope dilution procedure using P^{32} labeled red cells has been found convenient and reliable for the quantitative measurement of the residual blood in organs and tissues of farm animals. In radioisotope investigations and specific kinetic studies with blood-borne substances these values require emphasis. Because of the intimate relationship between the cells of the body and the blood, inherent errors in analysis may be encountered due to residual blood trapped in the tissues at the time of slaughter. For the most part, routine sampling is made from animals that have been bled out; therefore, total organ blood volume(6-8) was not considered a necessity in this study. It is pointed out that since the residual blood calculations are based upon total blood volume values, consideration should be given to some of the more important sources of error when the isotope dilution procedure is employed. These errors invariably leading to a high blood volume value include: a) loss of label from cells injected, b) non-quantitative injection of cells into the blood stream and, c) removal of label from circulating blood following administration. Every precaution was taken in this study to avoid or to correct these errors, and by management practices to avoid physiological changes known to affect the normal animal. Since whole organs could not always be analyzed conveniently, multiple samples were taken routinely from similar areas of the various organs and processed immediately. The percent of the total blood volume for individual organs showed considerable variation, but residual blood per gram within tissues was remarkably constant. There was a general tendency for a lower residual blood concentration in tissues and organs of older and heavier animals, and a decrease for muscle tissue with increased age

TABLE IV. Residual Organ Blood Volume of Swine at Various Weights.

Wt (lb)	Total blood vol (ml/100 g)	ml blood/100 g (fresh wt)								
		Lungs	Spleen	Kidney	Liver	Adrenal	Heart	Pancreas	Loin muscle	G. muscle
8	7.1	26.2	13.1	6.5	8.1	5.0	4.0	2.2	2.2	1.0
7	7.4	27.8	12.3	6.3	7.8	6.0	4.6	2.1	2.2	1.2
10	7.0	25.2	12.5	6.8	8.3	5.7	3.3	1.7	2.2	1.0
12	6.9	24.8	12.2	5.3	6.4	6.3	3.1	2.5	2.1	.9
Avg 9	7.1	26.0	12.5	6.0	7.7	5.8	3.8	2.1	2.2	1.0
± 2*	± .2	± 1.5	± .4	± .7	± .9	± .6	± .7	± .4	± .0	± .1
28	6.8	22.9	15.1	7.1	5.1	5.4	3.4	2.1	1.4	.79
32	6.5	28.2	16.3	7.5	5.9	5.4	3.1	2.3	1.9	.80
78	6.4	23.4	12.5	6.9	6.1	4.0	2.8	2.3	1.2	.82
Avg 46	6.6	25.0	14.6	7.2	5.7	5.0	3.1	2.2	1.5	.80
± 30	± .2	± 3.1	± 2.2	± .4	± .6	± .8	± .4	± .1	± .4	± .02
644	3.3	23.1	9.9	5.4	8.4	3.6	3.2	1.9	.92	.89
668	3.1	24.8	10.0	11.1	7.3	5.4	4.1	2.5	.90	.73
547	4.0	24.1	10.1	10.7	8.2	4.4	3.5	2.4	.92	.82
Avg 620	3.5	24.0	10.0	9.1	7.9	4.5	3.6	2.3	.91	.84
± 71	± .5	± 1.	± .1	± 3.4	± .6	± 1.1	± .5	± .4	± .01	± .08

* Mean ± S.D.

and weight. Little difference between species was noted except that the spleen, liver, pancreas and gastrocnemius muscle of cattle and sheep retained more blood than did those of swine. The lower concentration of blood in muscle noted especially in aged swine may well have been due to the increased fat content of these animals.

Summary. The residual blood in organs and tissue, as a function of age and weight, for cattle, sheep and swine sacrificed by conventional methods was determined by the isotope dilution procedure using P^{32} labeled homologous erythrocytes. Organ weights and total blood volume compared favorably with literature reports. Neither age, weight nor species appeared to influence significantly the ml of tissue residual blood/100 g fresh weight, except that the spleen, liver, pancreas, and gastrocnemius muscle of cattle and sheep retained more blood than did those tissues of swine. There was a general tendency for higher residual blood concentrations in organs and tissue of younger animals, but these findings were not consistent. Adrenals, heart, pancreas, kidney, liver, pituitary and muscle tissue retained less than ten ml of blood/100 g fresh weight, while lungs and spleen retained more residual blood. It is suggested that these values may provide a basis for estimated corrections in quantitative investigations for distribution of blood-borne substances in farm animals.

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Effect of Insulin on Free Tumor Cells in Peritoneal Exudate and on Their Host.* (22161)

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Soon after discovery by Warburg(1) of the high rate of glycolysis in tumors, several attempts were made to inhibit growth of tumors by influencing their sugar metabolism. Thus, Silberstein *et al.*(2) reported delay or complete inhibition of tumor growth in tarred animals after insulin treatment. This inhibition of carcinogenesis by insulin was observed also under various experimental conditions by von Witzleben(3), Muenzner and Rupp(4) and by Risse and Poos (quoted by Bauer(5)). Moreover, Braunstein(6) recorded for diabetics with tumors, better glucose tolerance and decrease of blood sugar level following increase in tumor size. However, Bauer(5) could not detect any variations of sugar metabolism in cancer patients or any change in their condition by variations of sugar level. This discrepancy in results was perhaps responsible for the lack of further publication on this problem as a careful search of the literature did not reveal any recent data on insulin effect in tumor-bearing animals. However, some statistical research has been done on the association of cancer with diabetes suggesting a possible relationship between sugar metabolism and malignant growth(7, 8,9).

The method of free tumor cell culture in peritoneal fluid(10-12) provided a useful tool for quantitative estimation of the effect of various factors on tumor cell growth. The use of globin insulin assured more continuous and uniform changes in blood sugar level. This selection of technic and material enabled us to obtain some quantitative information about insulin action on tumor cells and on their hosts.

Material and methods. Tumors Sarcoma-180 and Sarcoma-37 were grown by serial intraperitoneal transfers in CFW (Carworth Farms) mice. All normal mice treated with insulin were of the same strain and age. Doses of 10^6 to 10^7 tumor cells from peritoneal exudate were inoculated intraperitoneally into mice. In each experiment, 20 mice remained untreated and 20 received daily intramuscular injections of insulin doses (0.25 to 2 units). Peritoneal exudates were obtained for total and differential cell counts on 4th and 5th day of tumor cell growth by technic previously described(10). For 10 mice of each series the percentage of mitoses in tumor cells was recorded in addition to differential counts. Similar examinations were carried out on exudate induced in 20 control mice by injection of normal tissue (liver, spleen) suspensions. In other series, inoculated and normal mice were treated simultaneously with daily insulin injections for 5 days and their survival span was recorded.

Results. Results of total, differential, and calculated tumor cell counts in peritoneal exudates from untreated and insulin treated animals are recorded in Table I.

The data of Table I show that average concentration of tumor cells was not influenced significantly by 3 insulin doses of 0.5 unit (series IA). It was decreased markedly by doses of 1 or 2 units (series 1B, 1C, 2A, 3A, 4A, 5A, and 6A). This effect was due to decrease either of total exudate cell number (3A and 5A) or of the proportion of tumor cells (2A, 6A) or to both (1B, 1C, 4A). This effect could be prevented by injection of massive doses of glucose immediately after insulin: the same dose of glucose alone did not induce any variations in cellular formula of exudate. Thus both total number of cells and number of tumor cells are apparently stimulated in the presence of both insulin and glucose. Percentage of mitoses in tumor cells of controls was 9 to 19 (average 12) and in in-

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TABLE I. Growth of Free Tumor Cells in the Peritoneal Cavity of the Mouse under Insulin Treatment.

		Treatment with insulin		Results (averages and variation extremes)			
Exp. No.	Tumor inoculated, i.p.	Days	Units daily	Amt of peritoneal exudate	No. of cells (tumor & leukocytes)/mm³	% tumor cells	Calculated No. tumor cells/mm³
1 A	S- 37	3	.5	3+ 2+-3+	118 53–191	46 32–60	55 27– 88
B		3	1	2+ +-2+	92 60–112	39 14–53	34 15– 49
C		3	2	2+ +-2+	46 32– 77	36 18–53	15 5– 23
D		0	0	3+ 2+-3+	123 72–157	52 41–78	55 43– 79
2 A	S-180	4	2	2+ +-3+	23 19– 28	21 11–26	5 3– 7
B		0	0	3+ 2+-3+	26 16– 27	53 23–75	16 4– 45
3 A	”	4	1	2+ +-3+	146 56–213	42 34–57	63 25–113
B		0	0	3+ 2+-3+	193 48–320	39 30–47	78 14–134
4 A	”	5	1	+ +-2+	69 51– 91	59 30–72	43 15– 65
B		0	0	3+ 2+-3+	109 75–142	68 57–80	77 44–114
5 A	”	3	2	2+ +-3+	117 70–173	28 11–55	31 8– 53
B		0	0	3+ 2+-3+	280 189–419	31 16–68	76 39–129
6 A	S-37	4	1	2+ +-3+	139 88–180	17 11–28	25 10– 39
B		4 1 cc 20% glucose daily	1	3+ 2+-3+	194 135-317	37 19–58	68 35–102
C		4 1 cc 20% glucose daily	0	3+ 2+-3+	84 36–188	38 25–62	32 10– 68
D		0	0	3+ 2+	105 39–156	31 10–50	35 11– 56

20 mice in each group. For total numbers and differential counts, averages and extremes are indicated. Amount of exudate estimated* as proportionate to volume of fluid obtained by insertion of glass capillary in peritoneal wall: 3+ = .5 mm³ or more, 2+ = <.5 but >.1 mm³, + = <.1 mm³, 0 = no fluid.

* Reliability of this estimate was shown by comparison with the amount of exudate measured at autopsy.

sulin treated 0 to 11 (average 5). No significant variations (more than 10% of the average) in the leucocyte formula were noted in the exudate induced by normal tissue suspension.

Effect of insulin on host of tumor growth was estimated by comparing the life span in 3 groups of mice: 1) tumor-bearing and untreated, 2) tumor-bearing and insulin treated and 3) insulin treated normal mice. The results are reported in Table II.

It appears from Table II that mice of group 2 died earlier than in control groups 1 and 3. Administration of glucose after insulin decreased mortality rate in group 3, but not in group 2. Doses of 0.25 unit of insulin were insufficient to influence survival span of mice.

Analogous experiments were carried out in mice bearing subcutaneous tumors with similar findings but with a wider range of variations.

Discussion. Decrease in number of free tumor cells in the exudate after insulin treat-

TABLE II. Survival of New and Tumor-Bearing Mice Treated with High Doses of Globin Insulin.

	Mice with tumor growth					Mice without tumor growth (new mice)			
	Treated daily with requisite doses (units) of insulin								
	Group 1	Group 2				Group 3			
Days after 1st insulin inj.	Untreated controls (tumor growth, no insulin)	Tumor growth and insulin treatment				Insulin treatment, no tumor growth			
		.25	.5	1-2	1 + glucose	.25	.5	1	1 + glucose
3	20	20	18	15	20	20	17	15	18
5	19	18	12	13	12	20	15	13	16
7	14	16	7	8	7	20	12	11	14
10	11	9	2	1	2	20	12	11	14

Mice were inoculated intraperitoneally with 10^6 S-180 cells 4 hr before 1st insulin injection. Insulin given 5 successive days. 20 animals in each series. Dose of glucose: 1 cc of 20% solution. Number of surviving mice indicated.

ment could neither be interpreted as effect of dilution, since volume of exudate was reduced (Table I), nor as increase in proportion of leucocytes, because insulin did not modify the cellular formula of exudate in controls. Moreover, decrease in percentage of mitoses indicated the effect of some factor in the peritoneal fluid on the floating tumor cells. This factor was either insulin or some metabolic product of hypoglycemia, since glucose prevented cell damage.

Growth inhibition of tumor cells by insulin excluded obviously the possibility of incriminating malignant growth for early death of their hosts; on the other hand, comparison with results in insulin treated normal mice pointed out that sensitivity to insulin was increased by occurrence of malignant growth. Since the lethal effect of high insulin doses was partly reversible by glucose in normal mice and irreversible in tumor hosts, it may be presumed that in the latter some vitally important tissues were damaged by hypoglycemia and its products, or by insulin itself; for instance, it may be presumed that insulin increased cell permeability(13,14) for some toxic metabolites of malignant growth. Since combined treatment with insulin and glucose induced far greater effect on exudate and tumor cells than simple reversal of insulin action, a further study of this phenomenon may offer new insight in our problem.

Summary and conclusions. CFW mice were inoculated i.p. with S-180 or S-37 cell suspensions and treated with 3 daily i.m. injections of globin insulin (0.5 to 2 units). On the 4th or 5th day, specimens of peritoneal exudate were withdrawn from these mice and from untreated controls; the number of tumor cells per cmm and the percentage of their mitoses were consistently lower in insulin treated animals; this effect of insulin could be prevented by repeated injections of glucose (1 cc of 20% solution). In other experiments, the survival span was recorded for insulin treated tumor-bearing animals, their untreated controls and insulin treated normal mice. The first group showed the shortest survival which could not be extended by injections of glucose. It is concluded that growth of free tumor cells in the exudate was reversibly inhibited by hypoglycemia, its metabolic products or by the direct effect of insulin on tumor cells (permeability?) and that the same factors (or some of them) provided additional stress to some vitally important tissues damaged by tumor growth.

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Antifibromatogenic Activity of 19-Nor-α-ethinyltestosterone in the Guinea Pig.* (22162)

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Both the progestational and antifibromatogenic potencies of 19-nor-progesterone (19-nor-P) are greatly superior to those of progesterone (P)(1,2). Likewise, the progestational potency of (19-nor-α-ethinyltestosterone (19-nor-ET)(3) has been found to be 5 times that of α-ethinyltestosterone (ET) (4). ET also is antifibromatogenic though about 15 times less than P(5,6). Because of the clinical importance of 19-nor-ET which is active by mouth(4) its antifibromatogenic activity was studied.

Methods previously described(2) were used. Because of slow absorption of ET (0.2 to 0.4 μg/sq mm of the pellet) 4 to 8 pellets were implanted subcutaneously. Absorption rate of 19-nor-ET was 1.6 μg/sq mm. Absorption rate of 19-nor-P also was greater than that of P (7 against 3 μg/sq mm(2,5,7).

Results with ET and 19-nor-ET are summarized in Table I. No prevention of estrogen-induced abdominal fibroids was obtained with the quantities of ET used. On the contrary, with the same quantities of 19-nor-ET the fibrous reaction was greatly diminished; the same occurred even with as little as 50 to 70 μg/day (see individual values in parentheses). With ET values of 4 to 7 may still occur with 150 to 210 μg/day(5). Thus the antifibromatogenic potency of 19-nor-ET is probably more than 4 times that of ET. It is noteworthy that the increase from ET to 19-nor-ET is of the same quantitative order as from P to 19-nor-P(2). However, 19-nor-ET is far less potent than 19-nor-P and even than P.

There was also a very pronounced increase in prevention of abnormal uterine weights

TABLE I. Antifibromatogenic and Antihysterotrophic Activities of ET and 19-nor-ET in Castrated Female Guinea-Pigs with Subcutaneously Implanted Pellets of Estradiol (Absorption: 47 to 50 μg day). Duration of experiment: 3 mo. For classification of results see (6,8).

Steroid tested Compound	μg/day	No. of animals	Fibrous tumoral effect*	Q_{3-3}†	Uterine wt, g
ET	71 (35–127)‡	10	6 ± .9‖	1.9	6.0 (2.7–8.7)
19-nor-ET	80 (49–126)§	14	2 ± .2¶	.3	2.6 (1.5–4.0)

* Arbitrary values for number and size of tumors, ranging from zero to 12(6).
† " " " *large* tumors only, per animal (8).
‡ 4 to 8 pellets.
§ ½ to 1 pellet.
‖ Individual values: 2-2.5-3-4-5.5-6.5-7.5-7.5-8.5-11.
¶ " " : (1.5–1.5)-1.5-1.5-1.5-1.5-1.5-(2-2)-2-2.5-(3-3)-3. In parentheses: animals receiving only 50-70 μg/day of 19-nor-ET.

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* Cordial thanks are due to: Professor Carl Djerassi, Wayne Univ., Detroit, for 19-nor-ET; Messrs. Ciba, Basel, for ET; Syntex, S. A., Mexico, for a generous supply of estradiol.

which dropped from 6 g with ET to 2.6 g with 19-nor-ET.

Summary. The antifibromatogenic potency of 19-nor-ET is about 4 times that of ET. 19-nor-ET is also greatly superior to ET in antihysterotrophic potency, *i.e.*, in preventing uterine increase due to estrogen.

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Effects of Hallucinogenic and Tranquilizing Drugs on Serotonin Evoked Uterine Contractions. (22163)

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Evidence has been presented to support the hypothesis that serotonin (5 hydroxytryptamine) which has been found in extracts of mammalian brain(1,2) plays a role in the physiology of the nervous system of invertebrates(3,4). While many physiological properties of serotonin have been characterized (5), its action on the central nervous system has not been defined. Shaw and Woolley(6) think that serotonin injected in the mouse cannot reach the brain. Since the hallucinogen, d-lysergic acid diethylamide (LSD), has been shown to antagonize the effects of serotonin, both *in vivo*(7,8) and *in vitro*(9), it has been suggested that serotonin antagonism may be important in the mechanism of LSD induced psychosis(9). However, mescaline and bufotenine(10) which are also hallucinogenic, do not antagonize serotonin activity *in vitro* (9,5). Furthermore, while many lysergic acid derivatives are not hallucinogenic, they retain the ability to antagonize serotonin-induced uterine contractions(7).

Methods. In the present experiments we used the uterus of spayed rats brought into oestrus by the injection of ovarian hormone. The rat uterus was suspended (at 28.5-29.5°C) in aerated de Jalon fluid (NaCl 9.0 g; KCl 0.42 g; $CaCl_2$ 0.06 g; $NaHCO_3$ 0.5 g; glucose 0.5 g/l). We studied the reactivity of the organ to serotonin creatinine sulfate, acetylcholine (ACh) and oxytocin before and after treatment with Frenquel (alpha-4-piperidyl diphenyl carbinol hydrochloride), chlorpromazine (10-(3-diethylaminopropyl), 2-chlorophenothiazine hydrochloride), reserpine, desmethoxy reserpine, Lergigan (N (2-dimethylamino-2-methyl-1-ethyl) phenothiazine hydrochloride), mescaline (3,4,5 trimethoxyphenyl ethylamine sulfate) and LSD (d-lysergic acid diethylamide). To make a comparative evaluation of the antagonistic powers as well as to compare the specificity of each drug tested, we determined the uterine reactivity to a series of increasing concentrations of contractile agents, (serotonin, ACh and oxytocin)' starting from a minimally active concentration then increasing it until the maximal uterine contraction was reached. Each dose was given 3 times. We accepted, as the stabilized value, the mean height of contraction when the variation between the 3 contractions was not greater than 10%. Then we again tested the reactivity of the uterus to the contractile agents after the hallucinogenic or

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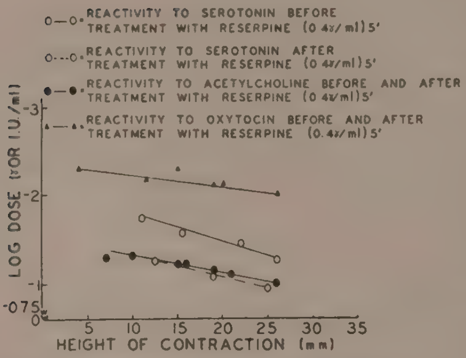

FIG. 1. Diminished contractility of uterus to serotonin after reserpine is contrasted to constancy of response to ACh and oxytocin. The figure discloses that to obtain an equal degree of activity after reserpine it is necessary to use a higher concentration of serotonin.

tranquilizing drug (Frenquel, chlorpromazine, reserpine, Lergigan, mescaline and LSD) had been left in contact with the organ for 3 to 5 minutes.

Results. The tranquilizing agents (Frenquel, reserpine, deserpidine and chlorpromazine) antagonize the serotonin-induced uterine contractions while in the same doses they display no antagonism either toward ACh or oxytocin. Lergigan and Meratran, similar in their chemical structure to chlorpromazine and Frenquel, respectively, do not display anti-serotonin activity in the concentrations at which they antagonize ACh. Successive washing completely reversed the antagonistic effects of Frenquel and of chlorpromazine after intervals of 30 and 60 minutes respectively. With reserpine and its analogue (both in concentration between 0.4 to 1 mg/l), the antagonism was long lasting; the uterine contractility being restored to normal values after 240 minutes.

Fig. 1 shows the uterine response to serotonin, ACh, and oxytocin before and after treatment with reserpine (0.4 mg/l) for 5 minutes. The negative log of concentration (γ/cc) of the contracting agent (serotonin, ACh and oxytocin I.U./cc) was placed on the ordinate axis, and the mean height of contraction (in mm) attained with each dose on the abscissae. Thus we obtained a graphic representation of the reactivity of the uterus to the contracting agent. No changes were observed in the response to oxytocin before and after treatment with reserpine. The graphical expression of uterine reactivity to ACh both before and after treatment practically coincides showing that no changes were induced by the drug. The open circles represent the uterine reaction to serotonin; the upper line showing the pretreatment reactivity, and the lower one indicating the decrease in sensitivity following treatment. The distance between these two lines represents the degree of inhibition exerted by reserpine on the serotonin-induced uterine contractions. Chlorpromazine (0.1 γ/cc for 3 minutes) and Frenquel (0.8 γ/cc for 3 minutes) increased the minimal active dose of serotonin from 12 γ/l to 60 γ/l and from 14 γ/l to 38 γ/l respectively.

Mescaline (Fig. 2) in concentration of 100 γ/l, facilitated the serotonin-induced contractions of the rat uterus. The increase of the serotonin contractions induced by mescaline is a reversible phenomenon. Mescaline alone in concentrations higher (over 0.4 mg/l) than that used in the above experiment evoked a uterine contraction. The contractile effect of mescaline was not modified by 240 γ/l of atropine sulfate. This concentration usually antagonized ACh-induced contractions of the rat uterus.

With LSD we were able to confirm the results of Gaddum(9), Cerletti(11), and Erspamer(5). As a matter of fact, using the comparatively high concentration of LSD of 1 γ/l (left in contact with the uterus for 5 minutes), we obtained a clear inhibition of the serotonin or mescaline-induced contraction,

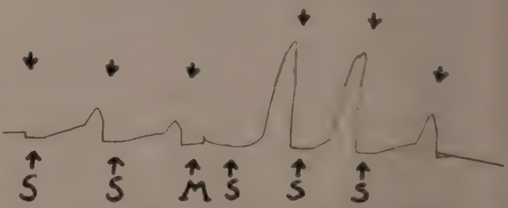

FIG. 2. Mescaline (100 γ/l) produces a transitory augmentation of minimal contraction evoked by serotonin (2γ/l). M and S indicate treatment of uterus with mescaline and serotonin respectively. In the concentration employed in this experiment mescaline by itself cannot cause a contraction.

while the activity of ACh was not affected. In contrast by using minimal effective doses of serotonin. we observed that LSD in low amounts (0.05 to 0.2 γ/l) left in contact with the uterus for 3 minutes increases the uterine response both to serotonin and mescaline. Since the action of LSD is long lasting, with the use of successive doses a summation of effect may occur. Thus inhibitory concentrations can be easily reached which prevent the demonstration of the facilitating action of LSD in low concentration.

Discussion. We were able to demonstrate that reserpine, Frenquel, and chlorpromazine are antagonists of serotonin activity in the rat uterus. Meratran and Lergigan, similar chemically to Frenquel and chlorpromazine respectively, do not exert a tranquilizing action in patients, and do not antagonize the serotonin-induced uterine contraction specifically. Mescaline and LSD, on the other hand, show a great affinity for serotonin receptors and under our experimental conditions, could be considered synergistic with serotonin. If an analogy is possible between the receptors in smooth muscle and the serotonin receptors in the brain, tranquilizing action seems to be associated with serotonin inhibiting properties while the hallucinogenic effect may be related to the facilitation of serotonin.

Summary. Tranquilizing drugs: Frenquel (0.8 mg/l), chlorpromazine (0.1 mg/l) and reserpine (0.4 mg/l), antagonize serotonin-induced uterine contractions, though they do not affect sensitivity of the uterus of the spayed rat either to ACh or oxytocin. The period of antagonism is longer lasting with reserpine than with chlorpromazine and is shortest with Frenquel. Mescaline (0.1 mg/l) causes a facilitation of serotonin activity, and in highest amounts causes a uterine contraction by itself. However, it is not antagonized by atropine (0.24 mg/l). A relatively high concentration of LSD (1 γ/l) antagonizes both serotonin and mescaline-induced uterine activity. In contrast low concentrations of LSD (0.05 to 0.2 γ/l) facilitate the effects of serotonin and of mescaline on the rat uterus.

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Occurrence in Plasma of an Extractable Lipid Mobilizer (LM)* (22164)

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Plasma from rats treated with cortisone, exposed to cold or subjected to the nephrotic syndrome prevented the delactescent action of heparin clearing factor *in vitro*(1). Hypercholesterolemia which was independent of adrenals or renal lesions was observed in rabbits prepared for the Shwartzman reaction and represented another example of the effect on lipids by non-specific stimuli(2). These observations suggested that concentration of a lipid mobilizer (LM) in the plasma could be increased either by cortisone or stress. In this paper we are reporting separation of such a substance from plasma of untreated and cortisone treated animals, also data which demonstrate the effect of LM on plasma lipids, food intake and body weight.

Materials and methods. Blood was collected in 0.1 M sodium citrate (1:9 whole blood) and centrifuged. The decanted plasma (2 ml/rat; 5 ml/human; 150 ml/dog) was dialyzed through Viscose Nu-Jax® casing against an equal volume of demineralized water for 6 hours at 25°C with constant agitation. Bulk lots were prepared from several liters of horse plasma under conditions that minimized bacterial and pyrogen contamination. The horse blood was collected aseptically and stored at 5°C for 72 hours to permit settling of the cells. The viscose casing was washed with 0.5% phenol and sterile, pyrogen-free demineralized water. The dialysis was at 25°C for 72 hours against sterile, pyrogen-free demineralized water in closed sterile containers. The dialyzate was concentrated at 75°C and 0.2 mm Hg pressure for 72 hours and freeze-dried for a similar time. The final product was sterile and did not produce fever in the animals used in the lipemia experiments. All animals were fasted 24 hours before blood samples were taken or before cortisone was administered. In the latter group, blood samples were obtained 4 hours after the administration of the cortisone. The LD_{50} of the dry extract was determined in Manor Farm All-Purpose mice, Wistar rats, guinea pigs, albino rabbits and mongrel dogs. A dose response curve was determined in normal Wistar rats and hypophysectomized albino rats. The effect of chronic administration of large

TABLE I. LM Activity of Plasma Dialyzates Prepared from Untreated and Cortisone-Injected Animals.

Species	Treatment†	No. of animals	No. of dialyzates showing LM activity	Hyperlipemic dose of dialyzate, mg/kg, I.V. in rats*
Human	None	10	10	285
Horse	None	4	4	300
	Cortisone	6	6	1
Dog	None	8	3	325
	Cortisone	5	5	1.85
Rat	None	10	10	300
	Cortisone	10	10	0.95
	Hypophysectomy Cortisone	10	0	>500

* Log spaced doses (10 rats/dose for each dialyzate; at least 100 rats per dialyzate).
† 5 mg/kg intramuscular.

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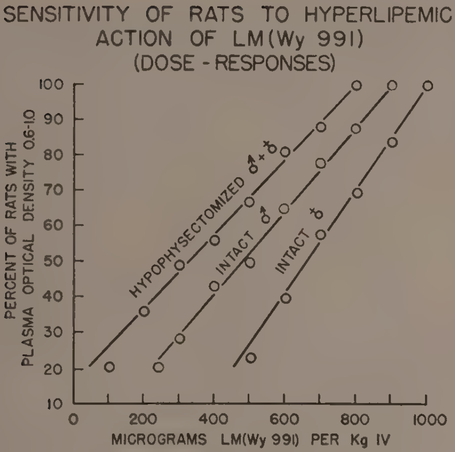

FIG. 1. Sensitivity of rats to action of LM.

doses of LM was studied in rabbits and dogs. At weekly intervals venous blood was analyzed for lipid phosphorus by the method of Fiske and SubbaRow(3), total fatty acids by the method of Stoddard and Dury(4) and total cholesterols by the Sperry-Schoenheimer technic(5). Optical density of the plasma was measured in a model B Beckman spectrophotometer.

Results. The LM activity of various plasma dialyzates when injected into rats is shown in Table I. Of the 63 dialyzates injected 15 failed to exhibit LM activity; these included 5 from untreated dogs and 10 from hypophysectomized rats. On the basis of mg of dialyzate/kg of body weight cortisone treatment caused an increase of approximately 300-fold in LM activity but on the basis of volume of original plasma the increase was approximately 1000-fold. LM activity was considered to be present when the optical density of the plasma of the injected rats increased from the initial 0.01 to at least 0.60. Plasmas exhibiting lipemia by this method also had at least a 50% elevation of total and esterified cholesterol, total fatty acids and lipid phosphorus. The dose necessary to elicit LM activity was 1/50 the LD_{50} or less. The intravenous LD_{50} of the dialyzates prepared from cortisonized animals calculated by the method of Bliss was as follows: (mg/kg) rats 48; guinea pigs 45; rabbits greater than 100; and dogs greater than 75. The injection of cortisone into hypophysectomized rats failed to cause the release of significant amounts of LM.

The dose response curves illustrated in Fig. 1 show that female rats were more resistant than males to the hyperlipemic action of LM and that intact rats were more resistant than hypophysectomized ones. In the hypophysectomized group females were as sensitive as the males. Each point on the curves represents 10 rats. The curves are qualitatively similar (no deviation from parallelism: $X^2 = 0.3$; $P>0.5$) but differ quantitatively ($F = 13.4$; $P<0.05$). The hyperlipemia resulting from injection of the ED_{100} (1 mg/kg) persisted for at least 6 hours and that from 60 mg/kg for at least 7 days. The degree of hyperlipemia following injection of a single dose of LM is shown in Table II. The error in the methods employed was smaller than the standard deviations. There was a prompt 2-fold increase of all plasma lipid components in the species tested. The hyperlipemia was invariably associated with increased optical density except in humans where there was no correlation. The ED_{100} in rats by intramuscular injection was 5 mg/kg. Intragastric administration of 100 mg/kg failed to produce hyperlipemia in rats. In conjunction

TABLE II. Mean Concentration (mg %) of Lipids in Plasma of Animals 1 Hr after Injection of LM.

Animal	No.	mg/kg	Total Ch Initial	Total Ch 1 hr	FA Initial	FA 1 hr	LP Initial	LP 1 hr	OD Initial	OD 1 hr
Mice	100	.5	53	174	148	450	5	12	.05	.90
Rats	475	.10	72	190	155	480	5	13	.04	.86
Guinea pigs	100	.50	66	185	160	440	5	12	.06	.80
Rabbits	40	.50	50	172	200	420	5	11	.05	.86
Dogs	20	1.	86	168	209	430	6	11	.05	.92

Ch = Cholesterol; FA = Fatty acids; LP = Lipid phosphorus; OD = Optical density.

TABLE III. Effect of LM (Mean Values mg %) on Plasma Levels of Lipids in 6 Dogs (60 mg Dialyzate/kg I.V. Once Weekly).

	Week of treatment									
	0	1	2	3	4	5	6	7*	8	9
Total Ch	83	165	190	220	245	240	210	240	202	189
Ester Ch	58	110	118	134	150	142	135	142	122	110
Total FA	212	440	520	680	590	540	606	630	420	250
Lipid P	7	12	12	14	13	13	12	13	10	8
Food taken daily (g)	265	370	540	550	550	552	550	550	500	450
Body wt (kg)	6	6	5	4	4	4	4	5	6	9

* LM discontinued.
Ch = Cholesterol; FA = Fatty acids.

with Dr. William Steiger, Dept. of Medicine, Temple University, 5 human subjects were injected intravenously with 0.25 to 1 mg dialyzate/kg. All responded with elevated plasma concentrations of cholesterol, fatty acids and lipid P of the same magnitude as the other species tested. None of the humans developed chills, fever, or other signs of toxie reactions.

Repeated single weekly injections of large doses of dialyzate containing LM in dogs and rabbits resulted in sustained hyperlipemia in which all elements of the plasma lipids were still elevated at least 2-fold 7 days following injection (Tables III and IV). The hyperlipemic animals developed marked hyperphagia in which food intake was 2-3 times that of control animals. The hyperphagic animals did not gain in body weight as rapidly as the controls and in some instances there was loss of body weight amounting to 33% of the initial. Discontinuing the administration of LM resulted in subsidance of the hyperlipemia and hyperphagia with an accompanying gain in body weight. Gross examination of dogs autopsied after 4 and 5 weeks of LM administration showed almost complete atrophy of the perirenal, omental, mesenteric and spermatic cord fat depots and marked depletion of subcutaneous abdominal fat. The livers were not fatty nor was there evidence of lipid deposition in other viscera or in the blood vessels.

TABLE IV. Effect of LM (Mean Values mg %) on Plasma Levels of Lipids in 20 Rabbits (60 mg Dialyzate/kg I.V. Once Weekly).

	Week of treatment					
	0	1	2	4	5	6
Total Ch	72	150	200	290	350	350
Total fatty acid	200	325	400	490	580	575
Lipid P	6	9	13	13	14	13
Food taken daily (g)	190	300	575	800	950	1000
Body wt (kg)	2.4	2.7	2.5	2.6	2.8	2.7

Ch = Cholesterol; FA = Fatty acid.

The anti-clearing (CFI) action of a dialyzate prepared from horses injected with cortisone is illustrated in Fig. 2. The *in vitro* method(1) utilized lipemic plasma and plasma from heparinized animals.

Discussion. The LM activity of the plasma dialyzate prepared from cortisonized animals was probably due to a mediator released by the injected cortisone. The dialyzate contained no cortisone or other steroids when it was subjected to infra-red analysis or to the Porter Silber and Liebermann-Burchard reactions. Cortisone requires conditions for dialysis which differ from those we employed(9). Assuming that all of the injected cortisone was absorbed and remained concentrated in the plasma and then entered the dialyzate, the amount injected was less than 10 μg/kg of body weight. Only 20% of rats receiving 5 mg of cortisone kg of body weight developed hyperlipemia in contrast to the 100% receiving 1 mg of dialyzate kg of body weight. Finally, plasma dialyzate from hypophysectomized rats injected with cortisone had no LM activity.

LM differed from the preparation of protamine available to us in being dialyzable and heat stable. Although LM and protamine(6) are CFIs *in vitro* and produce hyperlipemia when injected into animals other inhibitors such as diisopropylfluorophosphate (DFP)

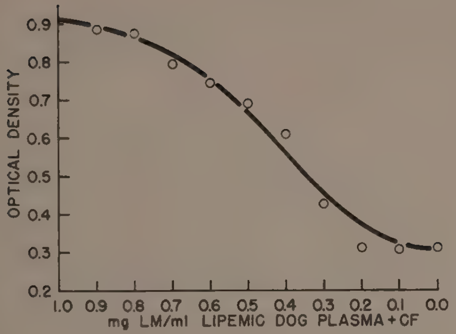

FIG. 2. CFI activity *in vitro* of LM plasma dialyzate vs. heparin clearing factor.

(7), and sodium cholate(8) failed to do so when administered in non-convulsant doses. It appears therefore that the hyperlipemic action of the dialyzate may not be related to its CFI action. The injection of LM into hypophysectomized rats produced hyperlipemia, whereas the injection of protamine failed to do so. Injection of 5 mg arginine/kg did not produce hyperlipemia in rats; larger doses resulted in fatality.

Growth hormone prepared from pituitary glands has been reported to be an active lipid mobilizer(10). LM differs from such preparations in being dialyzable, heat stable and in failing to promote growth in the presence of hyperphagia.

LM appears to resemble the factor extractable from the anterior pituitary which is free from growth-promoting or ACTH properties but is capable of mobilizing lipids(11). Several peptide fragments derived from the decomposition of the adipokinetic hormone are also capable of causing deposition of lipids in the liver(12). Our material, although dialyzable, is not a fragment produced by extraction procedures since the dialyzate without further treatment was capable of producing intense hyperlipemia.

Summary. 1. Freeze dried dialyzates of plasma prepared from dogs, rats and horses receiving cortisone contained a lipid mobilizer (LM) which markedly elevated plasma levels of free and esterified cholesterol, lipid P and total fatty acids when administered to fasted mice, rats, rabbits, guinea pigs, dogs and humans by intramuscular or intravenous injection. LM was not active by the oral route. The dialyzate also inhibited *in vitro* the delactescing action of heparin clearing factor. Plasma dialyzates prepared from untreated humans, horses, dogs and rats were 1/1000 as potent. 2. Cortisone injection failed to release LM in hypophysectomized rats. 3. LM activity of the dialyzate is not due to the same constituent responsible for CFI activity. 4. Hyperlipemia resulting from a single large dose persisted for at least one week in rabbits and dogs. 5. Repeated large doses of LM resulted in sustained and progressive hyperlipemia, hyperphagia and either loss in body weight or failure to gain. 6. Marked depletion of perirenal, mesenteric, omental, spermatic cord and subcutaneous fat were observed in dogs receiving weekly injections of LM.

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Effect of Growth Hormone on Sulfate Tm, Urea Clearance and Fasting Blood Glucose.*† (22165)

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Growth hormone has a variety of actions in animals(1): it promotes nitrogen storage; it produces diabetes mellitus; and more recently it has been shown to exert a renotropic action in increasing the reduced filtration rate, renal plasma flow and Tm_{PAH} of hypophysectomized dogs(2) and increasing these functions in intact dogs after more prolonged treatment(3).

During a recent study of the effect of diet and hormones on sulfate Tm, we observed that cortisone, as well as a low-protein diet, increased this value(4). Since cortisone tends to accelerate protein catabolism, and because of the intimate relationship of sulfate to protein metabolism, we have considered the possibility that sulfate Tm is influenced by a change in direction of growth hormone, as a protein-anabolic agent, on sulfate Tm. We have measured in addition the changes induced in glomerular filtration rate, renal blood flow and fasting blood glucose.

Methods. Observations have been made on 5 trained, unanesthetized female dogs maintained on laboratory diet which supplied approximately 60 to 100 g of protein daily. They were fasted 16 to 20 hours before an experiment and to insure hydration were given 700 to 1000 cc of water by stomach tube about an hour before the experiment. Glomerular filtration rate and (effective) renal plasma flow were measured, respectively, by clearances of creatinine and PAH which were administered intravenously by means of a constant-rate infusion pump. Na_2SO_4 was incorporated in the infusion fluid to give a concentration of 1% and sulfate Tm was determined at load/T ratios of 1.5 to 2. At low plasma levels of PAH this substance does not interfere with sulfate reabsorption(5). Urea clearance (at least three 15-minute periods) was determined at constant or decreasing urine flows about 2 cc/minute. Creatinine was determined by the method of Bonsnes and Taussky(6); PAH by the method of Smith *et al.*(7); sulfate by the method of Power and Wakefield(8); urea by the method of Seligson and Seligson(9); and whole blood glucose by the Nelson-Somogyi method(10). Three growth hormone preparations were used.§ Two preparations, A and C, were made by the Raben-Westermeyer method(11), and preparation B by the Wilhelmi method(12). Preparation A was in powder form and was dissolved in distilled water acidified to pH 3 with 0.1 N HCl immediately before use. Preparation B (Armour Lots Nos. R 491131 B, R 527094 B, R 491092 B) was supplied as a powder in sterile vials and was dissolved in water before use. Preparation C (G-25-32) was in sterile solution kept frozen before use. Growth hormone was given in single intramuscular injection in the afternoon on successive days, and measurements of renal function were made in the morning after the third injection. In a few instances the hormone was injected intravenously to determine its acute effect over the following 60 minutes.

Results. 3 mg/kg/day for 3 days of preparation B increased sulfate Tm 200% and 68%, respectively, in 2 out of 3 dogs (Table 1). (The third dog, No. 2, previously had shown an increase when given a low-protein diet for the first time, but not when placed on a low-protein diet a second time.) Plasma urea concentration and

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§ We are indebted to Dr. Maurice Raben of the New England Medical Center for preparation A, to Dr. Irby Bunding of the Armour Laboratories for preparation B, and to Dr. Herman Cohen of Princeton Laboratories, Inc., for preparation C.

TABLE I. Effect of Growth Hormone in 4 Normal Dogs.

Dog	Wt, kg	Het, %	Conditions	GFR,* cc/min.	RPF,* cc/min.	Sulfate Tm,* µM/min./kg	Plasma urea, mg/100 cc	Urea excretion, mg/min.	Urea clearance,* cc/min.	Blood glucose, mg/100 cc
2	16.3	38	Md†‡	61.9	238	8.3	32.0	12.9	40.5	89
	16.8	44	3 mg B†	78.2	307	8.0	15.0	7.8	50.6	137
5	12.2	44	Md	69.8	205	8.2	48.0	18.4	42.4	103
	11.7	44	3 mg B	86.0	267	24.5	11.1	6.6	59.3	102
4	12.0	37	Md	62.0	194	9.3	21.0	8.6	41.3	97
	12.5	38	3 mg B	80.7	240	15.6	6.7	4.0	60.1	146
	13.4	41	Md × 6 days	73.3	254	7.7	45.0	20.6	45.8	106
1	8.5	39	Md	32.5	92	9.1	32.0	8.6	26.7	93
	9.0	35	5 mg C†§	47.5	150	14.5	23.7	7.0	29.5	119

* Values for GFR, RPF, sulfate Tm and urea clearance are average of 3 periods.
† Md = mixed diet; B = B/kg/day × 3 days; C = C/kg/day × 3 days.
‡ This dog had just received 3 mg A/kg/day for 3 days without effect on renal function or blood sugar.
§ Lactation occurred in this dog with a dose of 3 mg/kg/day on third day, and again in a second trial with 5 mg/kg/day of preparation C after 3 days.

rate of urea excretion decreased in all 3 dogs. Plasma concentration decreased more than urea excretion in consequence of an increase in urea clearance of 25 to 47% over control values.

The filtration rate and effective renal plasma flow were increased by an average of 26% and 28%, respectively. In one dog, No. 5, blood glucose did not increase even though renal function was increased.

The two Raben preparations were less active than the Wilhelmi preparation in all respects. In dog No. 1, 3 mg/kg/day of preparation C for 3 days had very little effect on renal function or blood glucose concentration. Subsequently 5 mg/kg increased filtration rate and renal plasma flow but the effect on urea clearance and protein metabolism was slight (last experiment, Table I).

In experiments not reported here it was observed that 24-hour urine output, along with water intake, was often increased 3- or 4-fold after administration of preparation B. Moreover, during acute experiments listed in Table I, the animals treated with preparation B (in contrast to those treated with A or C) responded to a water load with a greater urine flow, resulting in greater recovery of administered water at the end of 2 hours (Table II). In view of the effect of water diuresis on urea clearance and urea excretion(13,14) we explored the possibility that diuresis in treated animals had increased urea clearance. This figure was therefore compared before and after hormone administration in the same dog at similar creatinine U/P ratios (Fig. 1). In each case the urea clearance was greater, at the same creatinine U/P ratio, after treatment, showing that diuresis was not an important factor.

In contrast to the effect of repeated administration of growth hormone intramuscularly, a single intravenous injection appeared to have no immediate effect. In 6 experiments on 4 dogs, a dose of 3 mg/kg of preparation B injected intravenously after 3 or 4 control periods produced no change in plasma urea concentration, urea excretion, filtration rate, renal plasma flow or sulfate Tm in the following 60 minutes.

TABLE II. Effect of Growth Hormone on Excretion of a Water Load in Normal Dogs.

Dog No.	Conditions	% water load excreted 1 hr	2 hr	GFR, cc/min.	Min creatinine U/P ratio
2	Md*	7	28	61.9	8.8
	3 mg B	17	62	78.2	8.2
4	Md	18	47	62.0	11.1
	3 mg B	19	70	80.7	9.8
1	Md	19	36	32.5	7.2
	5 mg C†	17	37	47.5	9.1
1	Low protein diet × 10 days	6	46	35.2	10.4
	Low protein diet and 1.3 mg B	35	99	49.7	9.0

* Md = Mixed diet; B = B/kg/day × 3 days; C = C/kg/day × 3 days.
† Dog lactating.

Discussion. Though the overall action of growth hormone is to spare protein(1), as exemplified by decrease in urea excretion in our experiments, the hormone tends to increase urea excretion at the expense of body urea in consequence of increased filtration rate. If protein metabolism remains constant, an increase in urea clearance will transiently increase excretion of urea until equilibrium is attained at a lower plasma concentration. In addition, since urea clearance increases with increasing urine flow(13), an increase in water intake and urine flow will further increase urea excretion at a constant filtration rate.

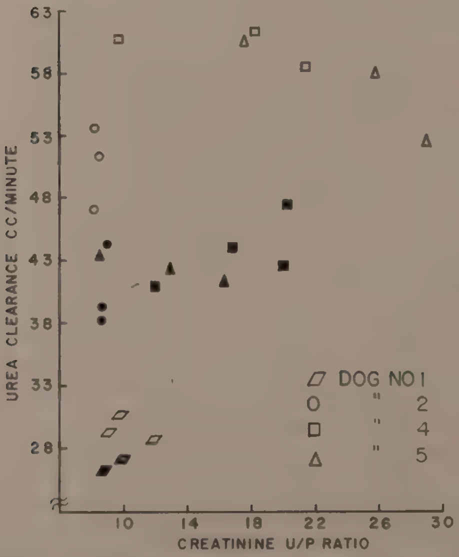

FIG. 1. Comparisons of urea clearances at similar creatinine U/P ratios before and after growth hormone in 4 dogs. Closed symbols, before treatment; open symbols, after treatment. Each symbol is a single clearance period. All observations were made at constant or decreasing urine flows.

Cortisone and growth hormone have, in general, opposite effects on protein metabolism, the first promoting protein catabolism, the second promoting anabolism; but both hormones increase sulfate Tm. Hence the effects on protein metabolism and on sulfate Tm are probably not directly related. The possibility remains, however, that some intermediate change in protein metabolism which may be induced by both hormones, such as, for example, a decrease in plasma level of one or more particular amino acids(15), induces changes in sulfate Tm.

It appears that renotropic action of growth hormone is one of its earliest observable effects. To induce diabetes mellitus, with similarly prepared extracts, either slightly larger doses have been used or injections have been continued for longer periods(16,17). Finally, it should be noted that the Wilhelmi preparation, which in our hands had greater renotropic and nitrogen-sparing effects than the Raben preparations, has been reported by Houssay and Rodriguez(17) to be more diabetogenic.

Summary. Growth hormone increased filtration rate, renal plasma flow, and urea clearance in 4 normal dogs, and sulfate Tm in 3 of these dogs, within 3 days. In 3 of the 4 dogs the fasting blood glucose concentration was elevated. Urea excretion (in short clearance periods) was decreased, but owing to the increased urea clearance, the plasma urea concentration decreased relatively more than urea excretion.

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Evidence for Participation of Vit. B_6 in Glutamine-α-Keto Acid Transamination-Deamidation Reaction. (22166)

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In a previous study(1) it was found that, although livers of vit. B_6 deficient rats exhibited markedly reduced glutamate-pyruvate transaminase and cysteine desulfhydrase activities, the glutamine-α-keto acid transamination-deamidation reaction proceeded at about equal rates with livers of control and experimental rats.† This finding, and our inability to resolve the glutamine enzyme by fractionation, were consistent with the concept that vit. B_6 was not involved in the glutamine-α-keto acid reaction, or alternatively, that affinity of the glutamine system for coenzyme was greater than that of the other enzymes studied. The present study supports the latter interpretation.

Patients given isonicotinic acid hydrazide (INAH) for treatment of tuberculosis developed a peripheral neuritis, ameliorated by administration of pyridoxine; such patients were also found to excrete in their urine greater than normal quantities of vit. B_6, apparently in a conjugated form involving INAH(2). These findings suggested to us that administration of INAH might represent a useful experimental procedure for depleting an animal of vit. B_6. We have therefore administered large doses of INAH to several groups of rats, and after periods ranging from 8 to 30 days, examined the livers for glutamine transaminase-deamidase activity in the presence and absence of coenzymes.

Methods. Weanling rats of Sprague-Dawley strain weighing 30 to 45 g, were used in Exp. 1 to 4, and adult Buffalo rats weighing 150 to 250 g were employed in Exp. 5 and 6. Animals were housed in individual cages and given water and diet *ad libitum*. *Exp. 1*: 26 rats were given stock Purina Chow diet; 13 of these were given 600 mg of INAH/k/day by gastric tube, and 13 received approximately same amount in drinking water. After

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† We have subsequently confirmed this observation using weanling rats of the Sprague-Dawley strain; adult Buffalo rats were used in the earlier work(1).

30 days, 5 animals of each group remained alive. *Exp. 2*: 30 animals received stock diet and 300 mg of INAH/k (by gastric tube) daily for 20 days; all survived. *Exp. 3*: 30 rats given vit. B_6 deficient diet and deoxypyridoxine as previously described(1), as well as 300 mg of INAH/k by tube per day for 14 days; 20 rats survived. *Exp. 4*: 30 rats were given same regimen as in Exp. 3 for 10 days; 25 rats survived. *Exp. 5*: 39 rats given stock diet and 480 mg of INAH/k by tube per day for 8 days; 26 survived. *Exp. 6*: 60 rats given stock diet and 480 mg of INAH/k by tube for 10 days; 37 rats survived.

Results. Large doses of INAH were employed because preliminary experiments indicated that smaller doses, at least over periods of 10-20 days, did not result in lowered transaminase activity. In general, rats given high doses of INAH were sluggish, developed moist snouts, and failed to gain weight or lost weight (0 to 0.7% body weight/day); death usually occurred shortly after INAH administration and was preceded by convulsions. Typical physical signs of vit. B_6 deficiency did not appear, even in Exp. 3 and 4, in which vit. B_6 deficient diet was administered; however, the duration of these studies was relatively short.

Enzyme assays were carried out essentially as described previously(1). The homogenate system consisted of 0.5 ml each of homogenate, L-glutamine (20 μM), sodium pyruvate (40 μM), and 0.1 M veronal buffer (pH 7.8), and incubated at 37° for 2 hours. The purified enzymes were isolated and lyophilized(3,4), and assayed in a system consisting of 20 μM of sodium pyruvate, 10 μM of L-glutamine, 10 mg of enzyme, in 0.4 ml of 0.025 M veronal buffer; the incubation period was 2 hours. Pyridoxal phosphate and pyridoxamine phosphate were used at a concentration of 100 γ/0.4 ml.

In Exp. 1, the livers of the treated rats exhibited greatly reduced glutamine-pyruvate transaminase-deamidase activity and increased glutaminase activity (Table I). Addition of pyridoxal phosphate or pyridoxamine phosphate increased glutamine-pyruvate activity in some instances, but not significantly in others. Because of the variable response to addition of the coenzymes, and because of the possibility that increased glutaminase activity might interfere with the determination of the glutamine-pyruvate reaction, subsequent studies were carried out using purified enzyme preparations obtained from pooled livers of a group of experimental rats and of their untreated controls.

TABLE I. Glutaminase and Glutamine-Pyruvate Transaminase-Deamidase Activity of Rat Liver Homogenates.*

Procedure	Sub-strate†	No. of rats	Range	Mean
Controls	Gl	9	.60–1.52	1.01
	Gl + P	9	2.95–4.54	3.56
INAH by tube	Gl	4	2.16–3.91	2.80
	Gl + P	4	.89–2.90	1.81
INAH in water	Gl	6	1.33–2.49	2.03
	Gl + P	6	.67–2.86	1.91

* Values are expressed in terms of μM of ammonia formed (corrected for blank ammonia) per ml of homogenate per hr; experimental details are given in the text.

† Gl = Glutamine; P = Pyruvate.

Experiments on several such purified enzyme preparations are described in Table II. These preparations did not possess glutaminase activity, and the effects observed with added coenzymes were readily reproducible. Preparations of the enzyme from livers of INAH treated rats exhibited a considerably reduced activity, and showed a significant increase in activity when pyridoxal phosphate or pyridoxamine phosphate was added. In

TABLE II. Glutamine-Pyruvate Transaminase-Deamidase Activity of Purified Liver Preparations.*

Exp. No.	Procedure†	Initial activity	Activity with coenzymes: Pyridoxal PO4	Activity with coenzymes: Pyridoxamine PO4
2	Con.	7.50	8.10	—
	Exp.	3.07	4.50	—
3	Con.	7.44	8.06	8.64
	Exp.	1.49	3.16	3.56
4	Con.	6.09	5.75	5.90
	Exp.	1.38	3.38	3.37
5	Con.	6.46	6.25	—
	Exp.	2.06	5.66	—
6	Con.	5.26	5.73	—
	Exp.	2.44	5.57	—

* See footnote, Table I.

† Con. = Control; Exp. = Experimental.

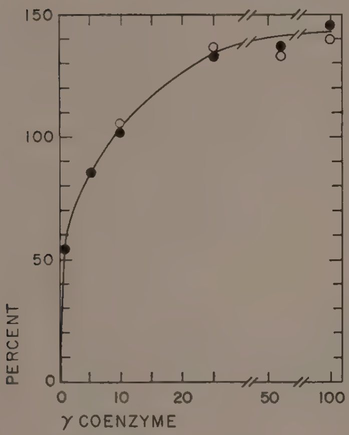

FIG. 1. Effect of pyridoxal and pyridoxamine phosphates on activity. Abscissa: γ coenzyme; ordinate: % increase in activity. Closed circles = pyridoxal phosphate; open circles = pyridoxamine phosphate.

confirmation of earlier studies(1,3,4) values of transaminase activity were in close agreement with those for ammonia formation. Maximal activation was observed with 25 γ of coenzyme (Fig. 1).

Discussion. The present results are consistent with the concept that the glutamine transaminase, like other transaminases, requires a vit. B$_6$ coenzyme. Failure of earlier attempts to demonstrate this may probably be ascribed to strong binding of the coenzyme by the enzyme. It is apparent that a relatively severe deficiency was required to remove vit. B$_6$ from this enzyme. It is of interest that concentration of coenzyme required for maximal activation of the partially resolved enzyme is of approximately the same order of magnitude as that required by other transaminases. It thus appears that deamidation of glutamine in this system may be made to depend on the presence of vit. B$_6$. It is probably the first step of the transamination-deamidation reaction (transamination of glutamine to α-ketoglutaramic acid) which requires vit. B$_6$. The second step, in which ammonia is formed, is hydrolysis of α-ketoglutaramate to α-ketoglutarate(5).

It has been reported that INAH inhibits *E. coli.* arginine decarboxylase(6), tryptophanase(7), and glutamate-oxalacetate transaminase of BCG(8); inhibition was reversed by pyridoxine, which is presumably converted to pyridoxal phosphate in the cell. It was also observed that patients being treated with INAH excreted unusually large amounts of xanthurenic acid in the urine after a test dose of tryptophan(2). The present results appear to provide general confirmation of the concept that this compound has an anti-vit. B$_6$ effect.‡ It is clear, however, that INAH has other effects (*cf*(9)) some of which may be related to the present observation of increased liver glutaminase and the fact that transaminase activity of preparations obtained from INAH-treated animals was often lower, even with added coenzyme, than that of controls. In 2 experiments in which INAH treatment was of relatively short duration, pyridoxal phosphate restored the activity to virtually control levels. (Exp. 5 and 6, Table II).

Beaton and Ozawa(10) reported that liver extracts of vit. B$_6$-deficient rats exhibited decreased ability to catalyze the glutamine-pyruvate reaction. Addition of pyridoxal phosphate to the extracts produced only very small and erratic activation.§ Our present results, which show a clear-cut reproducible *in vitro* effect of pyridoxal phosphate and pyridoxamine phosphate, lead to a conclusion which is not essentially different than that of Beaton and Ozawa, namely that vit. B$_6$ is involved in the mechanism of glutamine-α-keto acid transamination reaction. The present findings also suggest that INAH treatment may be a useful tool in the study of vit. B$_6$-catalyzed enzymatic reactions.

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‡ We have also found that livers of INAH-treated rats exhibited reduced glutamate-pyruvate and aspartate-ketoglutarate transaminase activities; the control level of activity was restored by addition of pyridoxal phosphate.

§ It was observed several years ago that livers of *very markedly* vit. B$_6$ deficient rats and mice exhibited reduced glutamine transaminase-deamidase activity. It may not be concluded from this evidence that the enzyme specifically required vit. B$_6$ because addition of pyridoxal phosphate did not increase the activity of enzyme preparations obtained from these very sick animals. (Meister, A., Morris, H. P., and Tice, S. V., unpublished data).

Summary. 1. Liver homogenates prepared from rats given large doses of isonicotinic acid hydrazide (INAH) exhibited reduced glutaminepyruvate transaminase-deamidase activity and increased glutaminase activity. 2. Purified enzyme preparations (free of glutaminase) prepared from livers of INAH-treated rats catalyzed the glutamine-pyruvate reaction at markedly reduced rates compared to controls. Addition of pyridoxal (or pyridoxamine) phosphate produced a considerable increase in activity of the preparations obtained from INAH-treated rats, but not of that of the untreated controls. 3. The evidence supports the concept that vit. B_6 participates in the glutamine transaminase system.

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Effect of Dihydrocholesterol and Soybean Sterols on Cholesterol Metabolism in Rabbit and Rat.* (22167)

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Because considerable evidence exists showing that such sterols as dihydrocholesterol and soybean sterols are absorbed from the gut, it was considered worthwhile to restudy the effects of these sterols on cholesterol metabolism in rabbit and rat. The data in this paper provide an explanation for the conflicting reports heretofore published and demonstrate the fallacy inherent in attempts to produce a negative cholesterol balance by inhibition of cholesterol absorption from the gut.

Experimental. Preparation of sodium acetate-1-C^{14}. Methylmagnesium bromide was carbonated with $C^{14}O_2$. Control diets consisted of Purina Rabbit Chow for rabbits and Purina Laboratory Chow for rats. Sterol diets were prepared by dissolving the requisite amount of dihydrocholesterol‡ or mixed soybean sterols§ in ether, adding the solution to control diets and evaporating the ether. Sera and tissues were prepared for sterol analysis as described previously(1). *Sterol determination.* Digitonin precipitable, Liebermann-Burchard (L.B.) positive sterols were determined by the method of Sperry and Webb(2) and total digitonin precipitable sterols by a modification of the Pollak-Wadler turbidimetric method(3). The latter method was modified as follows: Sterols were precipitated with digitonin in graduated glass-stoppered tube at 10°C. After precipitation the volume was adjusted in the tube, and one drop

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† This work was done during tenure of fellowship as Established Investigator of American Heart Assn.

‡ Dihydrocholesterol (Lot no. 105-220 I MRS), generously supplied by Schering Corp., contained 98% Liebermann-Burchard negative sterols.

§ The mixed soybean sterols (Control no. S-7577 and S-9707), generously supplied by Distillation Products Industries, contained 79% and 94% Liebermann-Burchard positive sterols respectively.

TABLE I. Recovery of Soybean Sterols from Acetic Acid Solution by Various Methods for Cholesterol Determination.

L.B. positive sterol added, mg		Representative recoveries of L.B. positive sterols							
		Methods							
		Sperry-Webb		Abell *et al.*		Modified Pollak-Wadler with			
						Acetone-ethanol		Acetone-methanol	
Cholesterol	Soybean sterols	mg	%	mg	%	mg	%	mg	%
.160	—	.170	106	.163	102	.163	102	.169	105
—	.160	.133	83	.150	94	.134	84	.162	101

of polyoxyethylene sorbitan mono-laurate (Tween 20) added to stabilize the suspension. Optical density of the suspension was determined photoelectrically. The digitonide precipitate was removed by centrifugation, and optical density of supernatant determined. Amount of digitonide was computed from the difference in optical densities. For a more complete precipitation of the soybean sterol digitonides it was necessary to change the alcohol in the precipitating medium from ethanol to methanol (Table I). *Feeding experiments.* Male New Zealand white rabbits weighing 2-2.5 kg were used. These were divided into 3 groups (Tables II and III). The first group received control ration. The second received 3 g dihydrocholesterol (DHC) 3 times weekly. The third received 3 g mixed soybean sterols 3 times weekly. Animals were maintained on these diets for 4 weeks at which time they were sacrificed and blood and tissue samples removed. Male rats of Sprague-Dawley strain weighing 200-300 g were divided into 3 groups. The first group received control ration, the second received 1% DHC (Table II), the third received 3% mixed soybean sterols (Fig. 1). Three animals from each group were sacrificed weekly for 5 weeks. Pooled liver samples were removed each time for determination of cholesterol synthesis and sterol content. The sterol contents of control and DHC groups were also determined after 8 weeks on the diet. Rabbits and rats were allowed to eat *ad libitum,* with weight gain determined weekly. There were no significant differences in diet consumption or weight gains between control and experimental animals. *Isotopic experiments.* Liver cell clusters(4) were prepared from livers of control and sterol-fed rabbits and rats; incubation with C^{14}-carboxyl-labeled acetate, isolation of cholesterol and analysis of its radioactivity were performed as described previously(5).

Results. The use of proper analytical procedure for sterol analysis was essential in this study. Soybean sterols, which consist largely of isomers of sitosterol and some stigmasterol, form a digitonide and give the L.B. reaction (6). We found that recovery of soybean sterols from solution by the commonly employed technics of Sperry and Webb(2) and Abell *et al.*(7) was incomplete. Table I shows the efficiency of recovery of soybean sterols by these methods as well as by the modified Pollak-Wadler procedure. The recovery of soybean sterols by the Sperry-Webb method and modified Pollak-Wadler procedure with acetone-alcohol was poor. The reason for the failure of recovery was solubility of soybean sterol-digitonin complex in 1:1 acetone-ethanol. In gravimetric experiments we have found that washing 28.4 mg of soy sterol digitonides with two 5-ml aliquots of 1:1 acetone-ethanol dissolved away 61% of the digitonides.

Recovery of soy sterols by the method of Abell *et al.* was better. However, the determinations in Table I were performed with a minimum exposure to the stream of air used in the method to evaporate petroleum ether extract to dryness. We have determined, in a manner similar to that employed by Sperry and Brand(8), that soybean sterols were 2 times as sensitive to air oxidation as cholesterol; like cholesterol, air oxidation destroyed soy sterols' ability to give the L.B. reaction. It is probable then that loss of soybean sterols during analysis by the method of Abell *et al.* resulted from air oxidation and

TABLE II. Liver and Serum Sterols of Rabbits Fed Dihydrocholesterol (DHC) and Incorporation of Sodium Acetate-1-C^{14} into Cholesterol by Excised Livers* of Rabbits and Rats Fed DHC.

Group	Animal	Diet	Duration of feeding, wk	Serum, mg % A Total digitonin precipitable sterols	Serum, mg % B Cholesterol†	Serum, mg % C DHC‡	Liver, mg/g A Total digitonin precipitable sterols	Liver, mg/g B Cholesterol†	Liver, mg/g C DHC‡	Specific activity of liver cholesterol§
I	Rabbit 1	Control	4	42	50	—	5.3	4.6	.7	—
	2	"	4	33	36	—	5.6	5.1	.5	306
II	3	9 g DHC/wk	4	100	72	28	21.3	12.7	8.6	34
	4	"	4	87	59	28	18.6	10.5	8.1	—
I	4 rats	Control	8	—	49	—	5.8‖	5.6	.2	387
II	4 "	1% DHC	8	—	30	—	8.4‖	5.2	3.2	127

* Each vessel contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer and 5 mg of $CH_3C^{14}OONa \cdot 3H_2O$ (5.1 mc/mM),

† L.B. positive sterol as determined by method of Sperry and Webb.

‡ Digitonin precipitable, L.B. negative sterol (column A minus column B).

§ Counts/min./mg of cholesterol/g dried liver.

‖ Determined gravimetrically as the digitonide.

that this loss may be far greater than shown in Table I. It should be added, however, that substitution of N_2 for air in the petroleum ether evaporation step in the Abell *et al.* procedure obviates this difficulty. From this evidence it seems clear that neither the Sperry-Webb nor Abell *et al.* procedures for cholesterol determination is adequate for analysis of soybean sterols. One may further conclude that use of these methods to determine serum and tissue total L.B. positive sterols may result in falsely low results when soybean sterols are present. For this reason reports, based on these methods, of a lowering of cholesterol levels after feeding soybean sterols may be suspect if soybean sterols are present in the body.

As the last column of Table I demonstrates, substitution of methanol in Pollak-Wadler type procedure provided the best recovery of soy sterols from solution.

Table II gives results of feeding experiments with DHC. It can be seen that DHC, which forms a digitonide but is L.B. negative (9), was found in sera and livers of rabbits and in livers of rats, thus indicating its absorption. Under these feeding conditions, that portion of the digitonin precipitable sterol which was L.B. negative was considered to be DHC. This assumption was strengthened by the fact that increased saturated sterol fractions(10) from the livers of rabbits 3 and 4 had infrared absorption spectra characteristic for DHC. It is of interest to note that the liver cholesterol content of rabbits 3 and 4 was elevated as well as the DHC content. This may have resulted from ability of liver to convert DHC to cholesterol(11). The last column of Table II demonstrates that livers of DHC-fed rabbits and rats have a decreased ability to incorporate labeled acetate into cholesterol. These results confirm the findings of Siperstein(12), Gould *et al.*(13), and Ivy *et al.*(14) that DHC is absorbed by the rat and further demonstrate absorption in the rabbit. In agreement with the report of Gould *et al.*(13) an inhibition of hepatic cholesterol synthesis in rats fed DHC has been demonstrated and a similar inhibition shown in the rabbit. The greater degree of inhibi-

TABLE III. Liver and Serum Sterols of Rabbits Fed Soybean Sterols for 4 Weeks and Incorporation of Sodium Acetate-1-C^{14} into Cholesterol by Their Excised Livers.*

			Serum, mg %			Liver, mg/g			
Group	Animal	Diet	Total digitonin precipitable sterols (methanol)	Cholesterol†	Soybean sterols‡	Total digitonin precipitable sterols (methanol)	Cholesterol†	Soybean sterols‡	Specific activity of liver cholesterol§
I	1	Control	26	25	—	5.7	5.6	—	607
	2		25	25	—	6.8	6.0	—	457
III	1	9 g soybean sterols/wk	40	45	0	10.2	5.1	4.6	155
	2		47	49	0	9.3	5.5	3.3	—
	3		32	35	0	10.0	6.3	3.2	242
	4		26	26	0	7.1	6.2	0.4	545

* Each vessel contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer and 5 mg of $CH_3C^{14}OONa \cdot 3H_2O$ (5.1 mc/mM).

† L.B. positive sterol as determined by method of Sperry and Webb.

‡ Digitonin precipitable (in methanol), L.B. positive sterol, corrected for cholesterol and for L.B. negative sterol content.

§ Counts/min./mg of cholesterol/g dried liver.

tion of cholesterol synthesis demonstrated in our animals undoubtedly reflects the longer feeding period for the sterol. Although atherosclerotic lesions of the aorta were not noted in our rabbits after a 4-week feeding period, it has recently been reported that a 0.5% DHC diet, fed to cockerels for 6 months, produces aortic atherosclerosis(15).

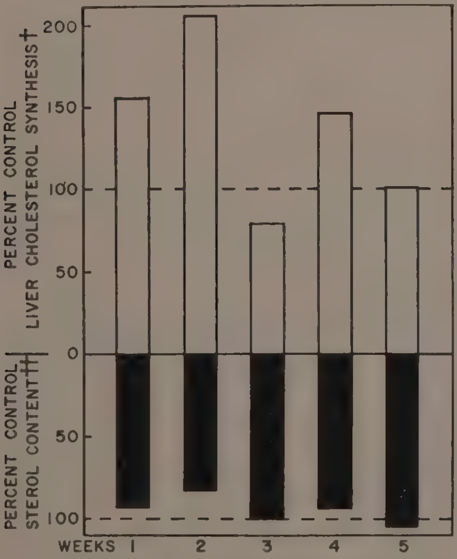

FIG. 1. Hepatic cholesterol synthesis and liver sterol content of rats fed a 3% soy sterols diet.

† $\dfrac{\text{C.P.M./mg cholesterol/g soy liver}}{\text{C.P.M./mg cholesterol/g control liver}} \times 100$

†† $\dfrac{\text{Total digitonin precipitable sterols/g soy liver}}{\text{Total digitonin precipitable sterols/g control liver}} \times 100$

Table III gives results of rabbit feeding experiments with soy sterols. It can be seen that the total liver sterols of rabbits 1, 2, and 3 of Group III are increased. As determined here, that fraction of total digitonin precipitable sterol (in methanol) which was neither cholesterol nor saturated sterol was considered to be soybean sterols. The validity of this assumption was demonstrated by the fact that infrared absorption spectra of the 1:1 acetone-ethanol soluble fraction of digitonides from livers of Group III rabbits 1, 2, and 3 were identical with infrared absorption spectrum of original mixed soybean sterols. It is of interest that rabbits fed soy sterols did not have measurable levels of these sterols in their serum. The last column of Table III shows that in livers of soy sterol diet rabbits 1 and 3 there was inhibition of cholesterol synthesis from C^{14}-labeled acetate. On the other hand Group III rabbit 4, which absorbed little soy sterol, showed a normal rate of cholesterol synthesis.

All rabbits receiving 9 g soy sterols per week for 4 weeks had atheromatous lesions of the aorta at autopsy. Infrared absorption spectra of the acetone-ethanol soluble fraction of the digitonides obtained from aortas of the rabbits in Group III also were identical with the spectrum of the dietary soy sterols. It is thus apparent that soy sterols are both absorbed and atherogenic.

Fig. 1 shows the effect of a 3% soy sterols diet on the liver sterol content and rate of hepatic cholesterol synthesis in the rat. It can be seen that moderate reduction in liver cholesterol levels produced in the first 2 weeks of diet was associated with a compensatory increase in hepatic cholesterol synthesis. This increased synthesis was sufficient to restore and maintain at normal the liver cholesterol levels for the next 3 weeks of the experiment. These findings are identical with results obtained when rats were fed large amounts of a cholesterol metabolizing *Escherichia coli* which destroyed biliary cholesterol passed into the gut(16). Negative cholesterol balance, produced by either steroid or non-steroid interference with cholesterol reabsorption, is counteracted by an increase in endogenous cholesterol synthesis.

None of the livers from rats fed a 3% soy sterol diet contained a measurable amount of soy sterols. There would thus seem to have been little absorption of these sterols. The transitory reduction in liver cholesterol seen in the first 2 weeks of the experiment undoubtedly resulted from competitive inhibition of cholesterol absorption by the non-absorbable soy sterols(17).

These data demonstrate absorption of soy sterols in rabbits. It has been shown that in rabbits soybean sterols produce a depression of cholesterol synthesis resembling that seen after feeding cholesterol(18), dihydrocholesterol, and other related sterols(12).

Discussion. It is possible to conclude from our data that there is no reason to expect a reduction of tissue cholesterol levels in man by the use of dihydrocholesterol and soybean sterols. The rabbit experiments further suggest that ingestion of such substances may be hazardous.

Summary. 1. Dietary dihydrocholesterol and soybean sterols were absorbed by rabbits, and soy sterols produced atherosclerosis in these animals. 2. Livers of rabbits and rats fed dihydrocholesterol and of rabbits fed soybean sterols had an impaired ability to incorporate C^{14}-labeled-acetate into cholesterol. 3. Rats fed a 3% soybean sterols diet did not absorb significant amounts of these sterols. Soy sterols failed to produce a lowering of total liver sterol levels in rats.

The authors are indebted to Dr. Nelson Ging for the infrared spectrographic analyses and to Dr. Harold Edelhoch for his helpful advice in modifying the turbidimetric procedure of sterol analysis.

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Inhibitory Effect of Protamine on Action of Intravenous Thromboplastin in Dogs.* (22168)

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Slow intravenous infusion of thromboplastin in dogs and other animals produced intravascular clotting resulting in deposition of fibrin on walls of blood vessels(1-7). With prolonged infusion, fibrinogen disappears from blood, while other clotting factors such as prothrombin and proaccelerin (plasma-Ac-globulin, labile factor) are only partially reduced(4,6). Animals recover and regain a normal fibrinogen level in 2-6 days(5-7). Rapid intravenous injection of thromboplastin induces formation of large thrombi near site of injection and causes prompt death of the animals(1,5,6). Attempts have been made with varying success to counteract the effects of thromboplastin *in vivo* with clotting inhibitors such as heparin(1,2), Congo Red, serum(2), soy bean trypsin inhibitor(3), alpha tocopherol phosphate and inositol phosphatide(8), and with thromboplastin splitting enzyme, thromboplastinase(9). Natural basic polypeptide protamine and some synthetic basic poly-amino acids were found recently to have antithromboplastic activity (10-12). They also accelerate clotting of fibrinogen by thrombin(12-14). However, when added to whole blood, coagulation is retarded as their antithromboplastic activity predominates.

The present communication deals with the influence of protamine on defibrinating and lethal effects of intravenous thromboplastin.

Materials and methods. Thromboplastin. This term refers to an extract in 0.9% NaCl of homologous, acetone dehydrated brain prepared according to Ratnoff and Conley(6). The fresh extract was diluted to give clotting time of 13-14 seconds in one stage prothrombin assay of Quick using plasma of dog to be infused. Protamine was obtained from Nutritional Biochemicals Corp. Solutions of 5 mg/ml were made in 0.9% NaCl, pH 7.4. Healthy mongrel dogs of either sex weighing 5 to 17 kg were used. Sodium pentobarbital (3% solution in 0.9% NaCl), was injected intraperitoneally, 1 ml/kg. Anesthesia was maintained during experiments by further doses as required. *Administration of thromboplastin and protamine.* Surgical sterility was not maintained. Femoral artery and vein were exposed on both sides. In slow infusion thromboplastin was introduced into femoral vein from calibrated burette. The first 50 ml were infused at 2-3 ml/min and thereafter at 4-5 ml/min. Rapid injection of thromboplastin was given at approximately 1 ml/sec. At end of experiments, incision sites were sutured and 500000 units penicillin and 0.5 g streptomycin administered intramuscularly daily for 3 consecutive days. Protamine, when given simultaneously with thromboplastin, was infused into opposite exposed femoral vein at 1 ml/min. When protamine was given prior to infusion of thromboplastin it was injected at the rate of 2-3 ml/min. Whole blood clotting time. Blood samples were taken from femoral artery. Two aliquots of 1 ml were put in glass tubes at 37°C. Clotting time of blood in the second tube was recorded. Thrombin clotting time was employed as a quick indication of presence of fibrinogen. One ml of blood was added to glass tube containing 5 units of Thrombin (Upjohn) in 0.1 ml 0.9% NaCl. After careful mixing the tube was put into water bath at 37°C. Blood was considered depleted of fibrinogen if no clot formed during 3 minutes. *Determination of fibrinogen.* One ml blood was collected into solid oxalates(15) and plasma separated by centrifugation. The method of Ware *et al.*(16) was adapted for analysis of fibringen in 0.1 ml of plasma.

Results. Inhibition by protamine of defibrinating effect of thromboplastin. Eight

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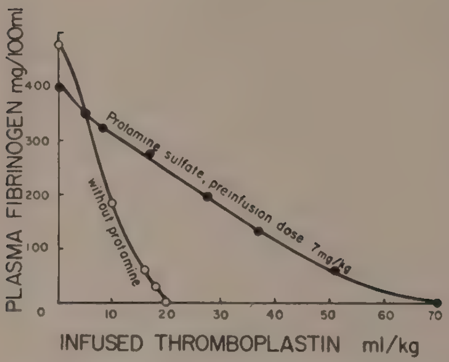

FIG. 1. Representative experiment showing inhibitory effect of protamine administered intravenously prior to infusion of thromboplastin on the decrease of plasma fibrinogen. Mean rate of thromboplastin infusion was 3.5 ml/min. An 8 kg dog was used.

normal dogs were given a slow infusion of thromboplastin, which resulted in a gradual decrease of their plasma fibrinogen level. Whole blood clotting times were elevated to 20-30 min soon after beginning of infusion as compared with 6-8 min in preinfusion samples. When the quantity of infused thromboplastin was near 20 ml/kg, blood became incoagulable. In some dogs a sudden drop in clotting time occurred in samples taken just before total defibrination, presumably due to presence of thrombin in circulation. Some of these samples clotted although they were mixed immediately with oxalate.

Ten dogs received protamine and were divided into 2 groups. The first group of 5 dogs was given 7-10 mg/kg protamine by slow injection; no untoward reactions or changes in plasma fibrinogen level or blood clotting time were observed with amounts and rates of injection employed. Thromboplastin infusion was started 5 min. after injection of protamine. Decrease in plasma fibrinogen level was markedly slowed (Fig. 1) and total defibrination required more than 3 times as much thromboplastin per kg as compared with dogs which did not receive protamine. Blood clotting times were similar to control dogs. Fibrinolysis could not be detected in spontaneously clotted blood at various intervals during thromboplastin infusion into protamine treated and control animals. Nor was lytic activity observed on addition of fibrinogen free blood, obtained at the end of infusion, to a clotted preinfusion sample of the same dog.

In the second group of 5 dogs the fibrinogen level was lowered to nearly half normal value by slow infusion of thromboplastin. Protamine was then infused. Fig. 2 shows, that plasma fibrinogen remained fairly constant during simultaneous infusion of both thromboplastin and protamine. There was no significant decrease in fibrinogen level even after infusion of protamine was terminated and infusion of thromboplastin was continued to a total of 70 ml/kg.

Effect of repeated infusions of thromboplastin and protamine. All defibrinated dogs recovered from the effects of slow infusion of thromboplastin (20 ml/kg) and their plasma fibrinogen returned to normal within a week. Several of these dogs were twice reinfused with thromboplastin at monthly intervals and approximately the same doses were needed for repeated defibrinations.

In another group of previously defibrinated dogs, protamine followed by thromboplastin was given one month later. The amount of thromboplastin (60 ml/kg), required for the second defibrination, was similar to that needed to produce first defibrination of protamine pretreated dogs (Fig. 1). After an-

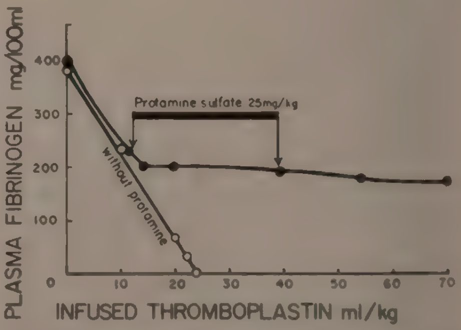

FIG. 2. Representative experiment showing arrest of plasma fibrinogen decrease resulting from administration of protamine to thromboplastin infused dog. Protamine infusion was started when 12 ml/kg thromboplastin had been given and terminated when the quantity of simultaneously infused thromboplastin reached 39 ml/kg. Mean rate of thromboplastin infusion was 4 ml/min. A 10 kg dog was used.

other month when only thromboplastin was infused, the usual dose of 20 ml/kg was again sufficient to render them fibrinogen free. These experiments indicate that repeated infusions did not cause development of either refractoriness or hypersensitivity to thromboplastin and that treatment with protamine did not produce any lasting resistance to the defibrinating action of thromboplastin.

Protecting action of protamine against lethal effect of rapid injection of thromboplastin. Rapid injection of thromboplastin of 5 ml/kg caused almost immediate apnea and death in 10 out of 14 dogs; 2 dogs died during the next 4 hours without recovering from anesthesia, while 2 more survived the injection. An illustrative experiment is described below: Respiration rate of 7 kg female dog under pentobarbital anesthesia was 18/min and pulse 120/min, clotting time 8 min and plasma fibrinogen 380 mg/100 ml. 35 ml of thromboplastin were injected into exposed femoral vein in 40 seconds. At end of injection breathing was rapid and shallow (60/min) then became sporadic and laboured. 75 seconds later breathing ceased and pulse rate was 15/min. At this time, a blood sample showed: clotting time 10 min, fibrinogen 285 mg/100 ml. 135 sec. after injection the heart stopped beating. Pathological examination revealed a blood clot in femoral vein, inferior vena cava, right atrium and ventricle and pulmonary artery, with marked dilatation of right heart. Protamine in doses of 7-10 mg/kg was slowly injected into 14 dogs. Five minutes later the dogs received a rapid injection of thromboplastin as described above. Soon afterwards some showed transient dyspnea, but none died. There was negligible decrease in plasma fibrinogen and recovery of dogs seemed complete. In one of 2 dogs sacrificed 10 days after injection, fibrin clots were found on wall of the right atrium. *Comment.* The present results show that protamine has the ability to counteract the effects of intravenous thromboplastin in dogs. It increases the amount of thromboplastin required for defibrination when injected prior to thromboplastin; it arrests the decrease of fibrinogen when administered simultaneously with thromboplastin and it protects animals against lethal effect of rapid injection of thromboplastin.

Inactivation of thromboplastin by protamine or by synthetic poly-arginine(17) *in vitro* is associated with the formation of a precipitate. This results from a salt-like interaction between basic guanidine residues of numerous arginine groups(18) of protamine and the acidic phospholipid components of thromboplastic protein(19). It may be assumed that a similar interaction of protamine with thromboplastin takes place *in vivo*.

Inhibitory effect of protamine infusion on intravenously administered thromboplastin suggests its application in clotting disorders which involve entry of thromboplastic material into the blood stream. Such a mechanism has been supposed to be the cause of afibrinogenemia in abruptio placentae(1,20), in amniotic fluid embolism(21,22) and in some cases of intrauterine death(23,24).

Summary. 1. Intravenous administration of protamine markedly increases the amount of slowly infused thromboplastin required for defibrination of dogs. Protamine given during infusion of thromboplastin, to a dog already partially depleted of fibrinogen, arrests further decrease in plasma fibrinogen level. 2. Intravenous administration of protamine protects dogs from the lethal effect of subsequent rapid injection of thromboplastin. 3. Mode of action of protamine and possible clinical utilization of its antithromboplastic properties are discussed.

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Variation in Reduced Glutathione in Bloods of Inbred Lines of Chickens.* (22169)

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There can be no doubt that the reduced glutathione (GSH) levels of blood and other tissues are influenced by the adrenal, thyroid, pituitary, pancreatic, and parathyroid hormones(1). There is a paucity of data, however, which evaluate the extent that activities of endocrine glands are under hereditary control and, therefore, the extent that variations in genetically controlled activities of endocrine glands regulate chemical activities of the cells.

Proof of genetic control of vital biochemical reactions in living organisms has been demonstrated principally by results of studies conducted on monoploid microorganisms(2,3). In higher animals, such evidence has resulted from studies of physiological abnormalities, primarily in the human being, from studies of pigment development and body coloration, or the presence or absence of a chemical constituent such as atropinase or a β-globulin in the sera(4-8). Williams *et al.*(9) have suggested a quantitative individuality in metabolism of humans and rats on the basis of variation in patterns of excretion of metabolites in urine. Breed differences in plasma or serum alkaline phosphatase in chickens(10), in thyroxine secretion rate of chickens(11,12), and in total body glutathione concentration in 2- to 14-day-old chicks(13) lay a broad basis for the study of biochemical genetics in chickens, but the majority of such studies has not dealt with these biochemical factors as bases for biological individuality.

Genetically controlled individual quantitative differences in biochemical constituents should tend to become fixed by inbreeding and, as a result of this increase in intra-line genetic uniformity, differences between inbred lines become more easily demonstrable. With availability of inbred lines of chickens within a single breed (White Leghorn) in our

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laboratories, studies have been initiated on constancy of and genetic control of the concentration of GSH in mature chickens.

Methods and materials. Blood levels of reduced glutathione have been determined for mature male and female birds of 3 inbred lines, *viz.*, Lines 22, 23, and 24 of White Leghorns, maintained by the Poultry Husbandry Department, A. and M. College of Texas. These birds were hatched during 1952, 1953, and 1954 seasons. All these lines were inbred to about the same extent. The average inbreeding coefficient of birds used in these experiments was 0.53. Whole blood GSH was estimated by the method of Grunert and Phillips(14). The method was modified by increasing the volume of hemolyzing saponin solution to 4 ml for each ml of blood. Using potassium oxalate as anticoagulant, blood samples were collected by venepuncture and were placed immediately into ice bath at about 0°C. Duplicate analyses were made on samples within 8 hours after collection. Hemoglobin values were determined colorimetrically as oxyhemoglobin and red cell volumes were estimated after centrifugation of blood samples for 45 minutes at 1400 x *g* in Wintrobe tubes.

The data were statistically analyzed according to the methods outlined by Snedecor(15) to evaluate the significance of observed differences. Repeatability estimates were calculated as product-moment correlation coefficients between results of successive determinations. Coefficients of variation were calculated as standard deviation divided by the mean and reported as percentages.

Results. Estimates of repeatability. One group of 18 males of Line 22, housed on the range, had a mean GSH level of 48.8 ± 1.8 mg/100 ml blood. Analyses made on the same birds 5 weeks later showed a mean value of 51.8 ± 1.8 mg/100 ml. The correlation or estimates of repeatability between these successive determinations on the same birds was found to be 0.58 and is statistically significant ($p<0.05$). The coefficient of variation for the first set of determinations was 15.8% and 14.4% for the second. In a second group of males from the same line, the mean GSH levels observed from subsequent determinations on 40 birds were 50.9 ± 1.1 and 63.0 ± 1.4 mg/100 ml blood, respectively. Coefficient of variation was 13.6% in the first, and 14.4% in the second determination. Again the estimate of repeatability between these determinations was found to be 0.58, a correlation which was highly significant statistically ($p<0.01$). Just prior to the second determination, the 40 males described above were moved from one set of open-air quarters to another. In view of the fact that no dietary change was made, it might be possible that the increased level of GSH observed in the second determination could be due to environmental changes. Regardless of whether the difference between means was the result of environmental effects or a possible uncontrolled analytical error, the significant correlation between determinations was indicative of an individuality in the level of blood GSH within an inbred line.

TABLE I. Repeatability Estimates of Reduced Glutathione Levels in Blood of Lines 22, 23, 24, and 22 × 24 Females. Ten birds in each series.

Line	Date	Avg glutathione, mg/100 ml	Estimate of repeatability*	Coeff. of variation, %
22	3/17/55	33.4 ± 1.6†		14.82
	26	39.5 ± 2.1	.90	16.63
23	17	21.7 ± 1.0		15.18
	26	25.7 ± 1.1	.78	13.90
24	17	26.9 ± 1.6		18.83
	26	33.0 ± 1.8	.88	16.89
22 × 24	18	29.0 ± 1.1		11.79
	29	34.9 ± 1.9	.79	16.88

* Significant at .01 level of probability.
† Stand. error of the mean.

Subsequent GSH determinations made on 10 mature females from each of inbred White Leghorn Lines 22, 23, and 24 were used to calculate estimates of repeatability of GSH measurement in the inbred female. In addition, successive determinations were made on 10 females produced from a cross of Line 22 males on Line 24 females. All of these birds were individually caged. Repeatability estimates for all these lines were highly significant (Table I) and ranged from 0.78 to 0.90. Again an increase of 18 to 23% in the aver-

TABLE II. Average Blood Levels of Glutathione of Mature Males from Inbred White Leghorn Lines 22, 23, and 24.

Line	Avg glutathione, mg/100 ml blood 1952	1953	1954	3 yr avg	No. of birds
22	51.7 ± 1.1	54.4 ± .9	51.5 ± 2.8	53.3 ± .7	85
23*	44.4 ± 1.6	41.2 ± 1.9	43.3 ± 2.0	43.0 ± 1.1	18
24	56.7 ± 1.1	50.3 ± 1.3	53.8 ± 2.0	52.2 ± 1.1	59

* Highly significantly lower ($p < 0.01$) than other two lines.

age GSH level in the second determination was noted. When the second GSH analyses were made, the environmental temperature was much lower than temperatures existing during first experimental period and possibly may account for the differences. While environmental effects could have affected the level of GSH, the rank order of levels in females as with the results with males, described above, was not appreciably altered.

Genetic Control. Levels of GSH were determined in 162 mature males of the 3 inbred White Leghorn Lines 22, 23, and 24. These birds ranged from about 10 to approximately 30 months of age when the determinations were made. The average levels, expressed in mg of glutathione/100 ml blood, are presented in Table II. These data show that average GSH levels of males from Line 23 were consistently lower over the 3-year period than levels of Lines 22 and 24. The hereditary control of the blood GSH level thus becomes apparent. The data were statistically analyzed for effects of age, inbred line, and dams and sires. Contrary to findings with cattle(16, 17), age was not a significant factor in determining the level of GSH in blood of these birds. The analysis did demonstrate, however, that the lower levels of GSH in birds of Line 23 were highly meaningful statistically ($p<0.01$).

The average GSH levels determined for 18 females from each inbred Line 22, 23, and 24, and 22 females from Line 22 x 24 crosses were 32.0 ± 1.2, 22.5 ± 0.8, 27.3 ± 1.3, and 28.7 ± 0.9 mg/100 ml blood, respectively. The differences between the 3 inbred lines were highly significant statistically. The significant line differences with both males and females provide strong evidence for the genetic control of quantitative differences in blood GSH. It is to be noted also that the levels in females were considerably lower than in males. The glutathione level observed for Line 22 x 24 females fell between the levels of parental lines. Thus, there was no evidence of a heterotic effect on GSH level. Unfortunately, representatives from other crosses between these lines were not available.

Effect of Erythrocyte Concentration. The erythrocyte is the primary source of GSH found in blood, and therefore, variation in number of red cells could account for part of the differences observed in GSH levels. In 52 males from Line 22 with a mean GSH level of 51.2 ± 0.9 mg %, the average hemoglobin content was 12.14 ± 0.23 g/100 ml blood. The correlation of 0.62 between concentrations of these 2 substances was highly significant statistically ($p<0.01$). Yet, in spite of the fact that the erythrocyte is the primary source of GSH in blood, differences in red cell number (indicated by hemoglobin content) accounted for only about 38% of the variation observed in the level of GSH of these males.

GSH levels, hemoglobin content, and red cell volumes were determined for a group of males and females from each inbred line. It is to be noted from the data (Table III) that GSH content of female bloods averaged from 26 to 34% lower than bloods from males of the same lines. However, the hemoglobin content was from 33 to 38% lower and the red cell volume was 32 to 37% lower in females. The major portion of the sex difference in GSH levels within a line was primarily the result of variation in number of erythrocytes as indicated by relative levels of hemoglobin and red cell volume. The decrease in total GSH due to reduction in volume of red cells in the female is partially offset by a 5 to 10% increase in GSH per volume of red cells. The average hemoglobin values and

TABLE III. Average Glutathione Levels, Hemoglobin Values, and Red Cell Volumes of Mature Males and Females from Inbred Lines 22, 23, and 24.

Line	Sex	Glutathione, mg/100 ml	Hemoglobin, g/100 ml	Red cell vol, %	Glutathione-hemoglobin ratio, mg/g	Glutathione conc. in red cells, mg/ml
22	♂	53.5 ± 1.6 (9)*	12.6 ± .5	41.9 ± 1.0	4.25	1.28
	♀	39.5 ± 2.1 (10)	8.4 ± .3	28.5 ± 1.0	4.70	1.39
23	♂	37.9 ± 1.0 (10)	12.3 ± .3	41.1 ± 1.1	3.08	.92
	♀	25.2 ± 1.5 (7)	7.9 ± .4	26.0 ± 1.1	3.19	.97
24	♂	48.8 ± 2.1 (9)	12.7 ± .4	42.3 ± 1.1	3.85	1.15
	♀	34.8 ± 1.1 (8)	7.9 ± .2	27.3 ± .8	4.42	1.27

* The number enclosed in parenthesis indicates the number of birds with all 3 values.

red cell volumes of the 3 inbred lines within sexes are essentially the same. Therefore, the observed line differences in GSH levels are clearly due primarily to different concentrations of GSH within red cells. It may be concluded that while environment and experimental treatment could have some effect, concentration of reduced glutathione within the erythrocyte is conditioned to a large extent by heredity.

Summary. 1. Significant correlations were obtained between successive reduced glutathione (GSH) determinations on the same birds. This was true in both males and females and indicated a high degree of constancy for the GSH level in individual chickens. 2. The blood level of GSH was determined in 162 mature males of 3 age groups from 3 inbred lines of White Leghorns. Age was not a significant factor, but one line had a highly significantly lower GSH level than the other 2 lines. Differences between the GSH levels in females from all 3 inbred lines were highly significant statistically. 3. Females of the 3 inbred lines had much lower GSH levels than the males. However, hemoglobin content and red cell volume of females were also much lower. Since the source of GSH is almost wholly the red cells, the difference in GSH level between males and females within a line could be attributed to relative numbers of red cells present in the sexes. Line differences in males and females appear to result from different concentrations of GSH within the red cell. The data provide evidence that the GSH concentration within the erythrocytes of mature chickens is to a very large extent genetically controlled.

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Cold-Adapted Animals. I. Changes in Blood Clotting and Electrophoretic Properties of Rabbit Plasma.* (22170)

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Changes in the clotting mechanism as a result of prolonged exposure to low temperatures, "cold adaption," have occasionally been noted(1) for non-hiberating mammals and people. More marked changes have been observed during hibernation or aestivation in mammals such as ground squirrel or hamster (1-4). During preliminary studies on cold adaption in rabbits, it was observed that plasma clotting times were prolonged, prothrombin times decreased, platelet material increased, and fibrin yield was greater. The present studies were undertaken to extend these experiments. Some changes in technic were made and earlier results, in qualitative agreement with the present ones are not included in the Tables. Electrophoretic studies, initially made to determine if abnormalities occurred in the fibrinogen component, have been included.

Methods. Conditioning of rabbits. Six young healthy male rabbits, about 3 kg, were placed in a cold room at 4°C for 2 months. During the first month animals were progressively clipped until hair remained only on the extremities. Animals were maintained in this condition by regular clippings for the last month. Animals gained weight at a slightly higher rate than controls and appeared healthy throughout the experiment. A similar number of controls were kept, unclipped, in the usual animal quarters. *Hematological technics.* 1. *Bleeding.* All glassware and needles were freshly siliconed before use (Desicote, Beckman Instrument Co.). Three ml of blood were drawn by cardiac puncture and discarded. A 50-ml syringe was placed on the needle, temporarily left *in situ,* and 40 ml blood was rapidly withdrawn. Thirteen ml of this blood was immediately transferred to paraffined 15-ml conical centrifuge tube, the remaining 27 ml transferred to 50-ml centrifuge tube containing 3 ml of 0.1 M sodium oxalate, and stirred by gentle inversion, using Parafilm as cover. 2. *Blood clotting times.* The 15-ml centrifuge tubes stood at room temperature for 10 minutes, and then tilted every 30 seconds until no flow occurred on complete inversion. 3. *Erythrocyte and platelet counts.* Portions of oxalated blood were diluted in Rees-Ecker solution and counted in a Neubauer counting chamber. 4. *Plasma clotting times.* The oxalated blood was spun for 5 minutes at 1500 rpm (133 g) and a portion of platelet-containing plasma withdrawn (samples "A" in Table II). Blood was then spun for 45 minutes more at 3000 rpm (1600 g) and platelet-poor plasma removed, (samples "B" in Table II). A portion of each sample was diluted with equal volume of 1% saline, and 0.1 ml transferred to 13 x 100 mm test tube and incubated for 150 seconds at 37°C. An equal volume of 0.0139 M $CaCl_2$† at 37°C was then added and the tube sat for

TABLE I. Platelet and Erythrocyte Counts, and Hematocrits of Normal and Cold-Adapted Rabbits.

Rabbit No.	Platelet counts (× 1000)	Erythrocyte counts (× 1000)	Hematocrits, %
	Normal rabbits		
993	322	5170	43.2
994	317	5930	44.5
995	417	5830	41.0
996	383	5960	41.6
997	389	4960	39.2
Mean	366	5570	41.9
	Cold-adapted rabbits		
976	395	5790	41.0
977	382	6730	47.4
978	485	6340	45.0
979	399	5210	43.9
980	487	5720	47.4
982	348	5620	45.5
Mean	416	5900	45.0
Mean % change in cold	+13.7	+5.9	+7.4

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† Contribution No. 2083.

TABLE II. Clotting Data on Blood from Normal and Cold-Adapted Rabbits.

Rabbit No.	Blood clotting time (min.)	Plasma clotting time (sec) A*	Plasma clotting time (sec) B*	Prothrombin time (sec)	Fibrin yield (g/100 ml)
		Normal rabbits			
993	26.5	87	173	66.0	.44
994	29.5	87	170	58.5	.52
995	24.5	77	150	47.0	.44
996	10.0	67	160	—	.43
997	25.0	77	400	57.5	.37
Mean	23.1	79.0	210.6	57.3	.44
		Cold-adapted rabbits			
976	29.0	87	273	—	.47
977	24.0	77	173	36	.59
978	21.5	117	733	38	.59
979	32.0	77	143	39	.60
980	38.0	93	263	32	.81
982	50.0	67	113	40	.66
Mean	32.4	86.3	283.0	37.0	.62
Mean % change in cold	+40.2	+9.3	+34.3	−35.4	+41.0

* Samples "A" spun at 1500 rpm (133 g) for 5 min.
" "B" " " 3000 " (1600 g) " 45 " .

50 seconds. It was tipped thereafter every 10 seconds, and the time for a firm clot to appear was taken as the end point. 5. *Fibrin determinations.* Clots from plasma clotting time determinations sat at room temperature for 16 hours, and then shaken free from walls of tubes, and the tubes filled with 1% saline. They were spun at 3000 rpm (1600 g) for 45 minutes and fluid above the collapsed clot drawn off through a capillary by suction. The fibrin mat was washed in this fashion 3 more times, and then analyzed for nitrogen by Micro-Nessler technic of Lanni(5). Conversion to protein was made by using the 6.25 factor. 6. *Prothrombin time.* These values were obtained in triplicate according to the one-stage method of Campbell, Smith, Roberts, and Link, as adapted by Holburn(6). Dilutions of platelet-free plasma were 5.0, 8.34, 12.5 and 20%, and values presented were taken from the log log plots of clotting time vs plasma concentration at the 10% plasma point. 7. *Total protein determinations* were done by the Micro-Nessler method of Lanni(5) on triplicate 0.2 samples of 1:100 dilution of plasma. 8. *Electrophoretic analyses* were made with a Perkin-Elmer apparatus using pH 8.6 Veronal buffer, ionic strength 0.1.

Results. From the data presented in Table I it appears that cold-adapted animals develop an increase in platelet[‡] and erythrocyte counts and in hematocrit.

Table II presents results of clotting studies. It can be seen that in cold adapted rabbits blood and plasma clotting times are increased, while prothrombin time decreased. There is also an increase in the fibrin yield.

The results of electrophoretic analyses are presented in Table III. Cold adaption resulted in decrease in albumin, and increase in β globulin and fibrinogen fractions. There also appears to be an overall increase in total protein under these conditions which although not statistically significant by these rigorous standards, was also present in preliminary studies. None of the serum patterns (results not presented) showed presence of any residual fibrinogen after clotting in either cold-adapted or normal animals.

[†] This concentration of $CaCl_2$ was used since it gave the shortest clotting time as determined in previous studies with rabbits.

[‡] Although the present data definitely suggests an increase in platelet count in cold-adapted animals, the statistical significance of the results might be questioned because of the poor reproducibility of this type of determination.

TABLE III. Relative Concentration of Proteins in Normal and Cold-Adapted Rabbits Based on Electrophoretic Data.

Rabbit No.	%					Total protein, g/100 ml
	Albumin	α-Globulin	β-Globulin	Fibrinogen	γ-Globulin	
			Normal rabbits			
993	68.8	8.8	8.3	9.0	5.1	7.01
994	64.4	9.4	10.0	8.6	5.6	7.30
995	66.4	8.5	12.0	7.6	5.7	7.48
997	65.2	9.0	10.0	8.5	7.5	7.20
Mean	66.2	8.9	10.1	8.4	6.0	7.25
			Cold-adapted rabbits			
977	58.6	8.4	14.8	11.8	6.3	8.03
978	53.7	8.0	15.8	14.0	8.7	7.18
979	61.0	6.6	13.4	12.5	6.6	7.30
980	56.0	8.2	13.6	15.0	7.2	7.93
982	57.6	9.5	13.5	11.6	7.9	7.38
Mean	57.4	8.1	14.2	13.0	7.3	7.56
$\frac{\Delta\ \%^*}{3S_D}$	2.08	.35	1.55	2.16	.33	.52

* Critical ratio—a value of 1 or greater indicates a probability of at least 99.7 that the means are significantly different.

Discussion. The observed increase in total protein (4.3%), erythrocyte count (5.9%), and hematocrit (7.4%) suggests that hemoconcentration occurred as a result of cold adaption. This effect has been observed by others(7,8) and evidence was presented which indicated that this phenomenon was of rapid development and short duration. Unfortunately we have no determinations of blood volume which would indicate whether the observed hemoconcentration was the result of reduced volume, or of increased quantities of the factors mentioned. Hemoconcentration, however, would not be expected to produce the observed changes in the clotting mechanism.

Increase in blood and plasma clotting time in the presence of decreased prothrombin time suggests that thromboplastin is less available in cold-adapted rabbits. Since there is an increase in platelet count it appears that platelets in these animals may be more resistant, although it is possible that thromboplastin is itself less effective for chemical reasons.

The decrease in prothrombin times in plasma from cold-adapted rabbits could be attributable to a number of factors, but may possibly be explained by increased fibrinogen shown by electrophoresis and increased fibrin yield. The prothrombin times are plotted in Fig. 1 as a function of fibrin yield. Sufficient correlation exists between these parameters to indicate that at least part of the observed change is due to the increased fibrinogen.

Summary. Rabbits maintained for 2 months at 4°C, developed increases in platelet and erythrocyte count, hematocrit, plasma protein concentration, β-globulins and fibrinogen and in whole blood and plasma clotting times. Decreases occurred in serum albumin concentration and prothrombin time. It was suggested that retardation of clotting in spite of decreased prothrombin time might be due

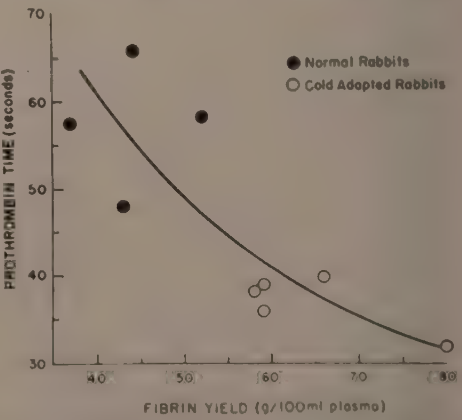

FIG. 1. Prothrombin times as a function of fibrin yield in all rabbits studied.

to an increase in resistance of platelets or to some chemical alteration of thromboplastin.

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Adrenal Corticoid Activities of 2-Methylhydrocortisone Acetate and 2-Methyl-9α-Fluorohydrocortisone Acetate. (22171)

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Biological activity of hydrocortisone is markedly increased by halogenation of C-9 (1-5) and by dehydrogenation at positions C-1 and C-2(6,7). When both modifications are present in the molecule, glucocorticoid, but not mineralocorticoid, activity of the steroid is further potentiated(8-10). This paper reports the results from biological testing of 2 new analogues of hydrocortisone: 2-methylhydrocortisone and 2-methyl-9 α-fluorohydrocortisone. These compounds have greatly enhanced gluco- and mineralocorticoid activities, as determined by subcutaneous administration on four different assays.

Materials and methods. Steroids. Steroid suspensions were made by grinding the compounds with carboxymethyl cellulose vehicle (8) in a ground glass tissue homogenizer. Compounds used were: 11 β, 17 α, 21-trihydroxy-4-pregnene-3,20-dione(F), 21-hydroxy-4-pregnene-3,20-dione, 21 acetate (DOCA), 9α-fluoro-11 β, 17α; 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (FF ac), 2-methyl-11 β, 17 α, 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (methyl F ac), and 2-methyl-9 α-fluoro-11 β, 17α, 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (methyl FF ac). The latter 2 new steroids were recently reported by Hogg, Lincoln, Jackson and Schneider(11). *Anti-Inflammatory Test.* This test measures the effect of steroids on granuloma formation in the rat by the cotton pellet assay described by Meier, Schuler and Desaulles(12) as modified by Dulin(9).

TABLE I. Anti-Inflammatory Assays Comparing Methylhydrocortisone Acetate, Methylfluorohydrocortisone Acetate, Hydrocortisone, and Hydrocortisone Acetate.

Compound*	Dose (mg/day)	No. rats	Mean granuloma dry wt (mg)	Potency ratio†
Vehicle	—	10	15	—
HA	.3	10	13.9	MA is
	.9	9	11.9	6.4 × HA
MA	.2	10	10.6	(3.4-10.7)
	.6	10	9.4	
Vehicle	—	8	14.2	
H	.3	8	11.7	MA is
	.9	8	8.4	4.5 × H
MA	.1	8	11.0	(2.6-7.2)
	.3	8	7.1	
Vehicle	—	10	15.3	
H	.2	9	10.6	MFA is
	1.0	8	6.3	9 × H
MFA	.02	9	9.4	(7.1-11.4)
	.1	8	7.3	

* HA = Hydrocortisone acetate; MA = Methylhydrocortisone acetate; H = Hydrocortisone; and MFA = Methylfluorohydrocortisone acetate.

† Figures in parentheses indicate limits of potency ratio at P = .95.

TABLE II. Comparisons of Methylhydrocortisone Acetate with Fluorohydrocortisone Acetate by the Liver Glycogen Technic.

Assay	Potency ratio* (methylhydrocortisone acetate/fluorohydrocortisone acetate)
1	.63 (.56– .71)
2	.97 (.80–1.17)
3	.85 (.65– .96)
4	.48 (.44– .54)
5	1.16 (.97–1.39)
Mean	.82

* Figures in parentheses indicate limits of potency ratio at $P = .95$.

Glycogen Deposition Test. The technic used was a modification(8) of the method of Pabst, Sheppard and Kuizenga(13). *Muscle Work Assay.* This assay was essentially that of Ingle(14,15) except that in one experiment electronic counters were substituted for mechanical work recorders. The steroids were injected in equal amounts twice in a 24-hour period. At the end of 24 hours, the experiment was terminated even though in some cases the rats were alive and still working. The work performed is reported in terms of revolutions of the counters. *Sodium Retention Test.* This test(8), a modification of the method of Marcus, Romanoff, and Pincus (16), measures the effect of compounds on sodium excretion in the adrenalectomized, salt- and water-loaded rat within four hours after administration. Since F is almost inactive on this assay(8), the reference standard was DOCA. Data obtained from these assays were pooled and submitted to an analysis of potency ratios with respect to DOCA. Potency ratios in this and the preceding assays were calculated by the method of Irwin(17).

TABLE III. Glycogen Deposition Activity of Methylfluorohydrocortisone Acetate.*

Compound	Dose (μg)	% liver glycogen
Hydrocortisone	300†	.5
	600	1.2
	900†	1.7
Methylfluorohydrocortisone acetate	5	.6
	10	.8
	15	1.3
	30	1.6
	45	1.9
	50	1.8
	90	2.4
	150	2.5

* Pooled data from 3 experiments.

† 10 rats/dose. All other values are means of 5 rat dosage groups. Calculated potency ratio is 38.0 × hydrocortisone. Limits of error are 97-104%.

Results. Table I presents data from anti-inflammatory assays comparing methyl F ac with F and F ac, and methyl FF ac with F. The potency of methyl F ac is calculated at 6.4 x F ac and 4.5 x F, while methyl FF ac is approximately 9 x F. By these same assay procedures, FF ac has been reported as being 7 x F(9).

Potency ratios of methyl F ac to FF ac in the glycogen deposition assay are given in Table II. The value of .82 represents the mean of 5 quantitative assays. Since FF ac has been found to be 13 x F by this assay(8), the potency of methyl F ac can be calculated to be approximately 10 x F. In Table III are presented data which indicate that methyl FF ac is 38 x as potent as F on this test.

TABLE IV. Comparative Activity of Methylhydrocortisone Acetate, Methylfluorohydrocortisone Acetate, and Hydrocortisone on the Muscle Work Assay.

Treatment	Dose	Revolutions*	No. rats
Untreated controls	—	8244	34
Hydrocortisone	1 mg	20050	13
Methylhydrocortisone acetate	125 μg	18630	16
	150	23280	14
	250	26500	4
	500	29680	4
Untreated controls	—	3895	10
Hydrocortisone	150 μg	6624	14
Methylfluorohydrocortisone acetate	10 μg	6247	13
	15	7921	10
	50	10427	6

* Mechanical counters were used in assaying methylhydrocortisone acetate, and electronic counters for methylfluorohydrocortisone acetate.

In the muscle work test, methyl F ac was 6-7 x as potent as F (Table IV). Between this assay and the evaluation of methyl FF ac, electronic counters were substituted for mechanical calculators on the Ingle Fatigue Apparatus and, therefore, the values obtained on the latter assay cannot be directly compared with those presented for methyl F ac. The data obtained from comparing methyl

TABLE V. Sodium Retention Activity of Methylhydrocortisone Acetate and Methylfluorohydrocortisone Acetate as Compared with Desoxycorticosterone Acetate.

Compound	Dose (μg)	No. rats	Sodium excretion (% of control)	Potency* (× DOCA)
DOCA	1	24	69	1.0
	2.5	6	59	
	5	12	48	
	10	126	48	
	20	12	35	
	40	12	27	
Methylhydrocortisone acetate	.44	6	90	2.6
	.88	6	68	(2.16–3.10)
	1.0	12	68	
	1.5	6	78	
	1.75	6	57	
	2.0	12	52	
	3.0	6	42	
	3.5	6	46	
	4.0	12	41	
	6.0	6	34	
Methylfluorohydrocortisone acetate	.001	6	87	90.0
	.01	18	79	(68–123)
	.1	24	41	
	1	18	24	
	10	6	24	

* Figures in parentheses indicate limits of error at P = .95.

FF ac with F on this assay are also presented in Table IV and indicate that this steroid has between 10 and 15 x the potency of F.

Comparison of potencies of methyl F ac and of DOCA by the sodium retention assay was difficult. Variation in results with methyl F ac made it necessary to conduct 5 multidose assays of this steroid. These data are presented in Table V. Methyl F ac is calculated at about 2.6 x DOCA. This steroid, therefore, has less mineralocorticoid activity than FF ac, which has been found to be 5 x DOCA on this assay(8). Data from assays of sodium retention activity of methyl FF ac are also presented in Table V and reveal the remarkable potency of this compound. Statistical analysis of the data shows methyl FF ac to have approximately 90 x the sodium-retaining potency of DOCA.

Discussion. Methylation of F at C-2 increases the mineralocorticoid and anti-inflammatory properties of this hormone to a lesser extent than fluorination at C-9, but these two activities are in about the same ratio in methyl F ac and FF ac. This is in contrast with the Δ^1 analogue of F in which the anti-inflammatory potency is increased out of proportion to the electrolyte activity(9).

The glycogen deposition activity of FF ac is 13 x F and that of 11 β, 17 α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione, 21-acetate (Δ^1F ac) is 3 x F; both changes in the molecule (Δ^1FF ac) resulted in this activity being 49 x F(8). The anti-inflammatory effect of

TABLE VI. Comparative Corticoid Activities of Methylhydrocortisone, Fluorohydrocortisone and Methylfluorohydrocortisone Acetates.

Assays	Steroids assayed Methylhydrocortisone acetate	Fluorohydrocortisone acetate	Methylfluorohydrocortisone acetate
Anti-inflammatory	4.5 × hydrocortisone	7 × hydrocortisone*	9 × hydrocortisone
Glycogen deposition	10 ”	13 ” †	38 ”
Muscle work	6-7 ”	not determined	10-15 ”
Sodium retention	2.6 × DOCA	5 × DOCA †	90 × DOCA

* Dulin(9). † Stafford *et al.*(8).

FF ac is 7 x F and that of Δ^1F ac is 3 x F: the Δ^1-9 α-fluoro analogue has 14 x the potency of F(9).

In these experiments, a methyl group at C-2 and fluorine at C-9 in the hydrocortisone molecule did not cause as great a potentiation of glucocorticoid properties as both Δ^1 and fluorine in the same steroid nucleus. Regarding mineralocorticoid potency, however, quite the opposite is the case. Δ^1F, like F, is almost inactive on the sodium retention assay used to evaluate these corticoids, and the Δ^1-9 α-fluoro analogue of F has no more mineralocorticoid activity than FF ac(8). Whereas substitution of fluorine for hydrogen at C-9 and methyl for hydrogen at C-2 each causes a great increase in sodium retention activity of F, the tremendous mineralocorticoid potency of methyl FF ac is considerably greater than would be expected on the basis of the electrolyte activity of methyl F ac or FF ac.

Summary. (1) Two new analogues of hydrocortisone, 2-methylhydrocortisone acetate and 2-methyl-9 α-fluorohydrocortisone acetate, have been tested for corticoid activity on the following assays: anti-inflammatory, glycogen deposition, muscle work, and sodium retention. A summary of the data is presented in Table VI. (2) The sodium-retaining activity of 2-methyl-9 α-fluorohydrocortisone acetate is 90 x desoxycorticosterone acetate and is, therefore, one of the most potent mineralocorticoids.

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Effect of Ultraviolet Radiation on Iron-binding Capacity of Plasma. (22172)

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In applying our recently developed methods for the determination of plasma iron(1) to ultraviolet-irradiated plasmas (pooled blood-bank ACD plasmas, "UVR-sterilized"), we found significantly lower values for the iron-binding capacity of these plasmas compared with non-irradiated samples. The present study was initiated to investigate this finding and to determine the extent to which siderophilin, the protein responsible for the specific iron-binding capacity of plasma(2,3) was affected by UVR.

Materials and methods. Plasmas obtained from blood-bank ACD-treated blood samples

TABLE I. Alteration of Siderophilin Iron-Binding Capacity with Irradiation.

Test material	UVR time (sec)	TIBC* (μg Fe/ml)	% TIBC remaining	C[†] $\times 10^2 \times$ sec^{-1}
(a) ACD plasma—α[‡] = 67, BI[§] = 0.98 μg Fe/ml	0	2.53	100	
	25	2.06	81.5	.82[‖]
	50	1.57	62.0	.95
	75	1.46	57.7	.73
	100	1.35	53.4	.63
	150	1.31	51.8	.44
(b) Fraction IV-7—α = 10.1, BI = 0.26 μg Fe/ml	0	3.91	100	
	10	3.03	77.5	2.5
	25	2.23	57.0	2.2
	50	1.37	35.0	2.1
	100	.86	22.0	1.5
	150	.84	21.5	1.0
(c) ACD plasma-Fraction IV-7 mixture—α = 56.7, BI = 0.29 μg Fe/ml	0	3.04	100	
	15	2.59	85.2	1.1[‖]
	25	2.22	73.0	1.3
	50	1.70	55.9	1.2
	100	1.41	46.3	.77

* TIBC = Total iron binding capacity of 1 ml of test solution.

† $$C = \frac{2.3}{t} \log \frac{\text{TIBC at } t = o}{\text{TIBC at } t}.$$

‡ $$\alpha = \frac{2.3 \times \text{optical density}}{\text{Light path, cm}} \times \text{dilution}.$$ Optical densities of appropriate dilution of test solutions of 1 cm depth were determined at 254 mμ with a Beckman spectrophotometer.

§ BI = Bound iron contained in 1 ml of test solution.

‖ These early values (0-20% siderophilin inactivation) are the least reliable since they are most affected by experimental error involved in determination of TIBC.

(1.84 g dextrose, 1.65 g sodium citrate, 0.6 g citric acid in 75 ml of water added to 500 ml of blood) and concentrated siderophilin, (plasma fraction IV-7, Cohn*), assaying 41% on the basis of its specific iron-binding capacity, were employed in this study. The irradiations were performed with an apparatus[†] having 2 Westinghouse cold-cathode Sterilamps as the UV light source. Their emission at 2537 A represents approximately 95% of the total UV output. A glass-bottomed, plastic-sided tray (31 x 6 x 1.3 cm) was centered 2.5 cm below the outer surface of the lamps and parallel to their longitudinal axes. The average integrated radiation intensity at this distance was 11.3 ± 1.1 milliwatts/cm^2 as measured by the uranyl-oxalate actinometric method(4). A 3-ml sample of the test solution was uniformly spread over the tray to a constant depth of 0.016 cm and exposed directly to the UV lamps at room temperature for exposure periods ranging from 10 to 150 seconds. Following the irradiation, each sample was removed from the tray and tested for its total iron-binding capacity (TIBC). The TIBC is a measure of the active siderophilin concentrations in plasma and represents the sum of both the iron-complexed and iron-free siderophilin species as determined by the bound iron (BI) and the unsaturated iron-binding capacity (UIBC) test methods, respectively(1). A decrease in the TIBC was considered a measure of siderophilin inactivation.

Results. Pooled plasma, fraction IV-7, and various plasma fraction IV-7 mixtures were irradiated according to the procedure described and their total iron-binding capacities determined. Table I presents the data obtained from typical experiments. It illustrates the quantitative relation between the amount of UVR received by the test siderophilin con-

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* Courtesy of Dr. D. M. Surgenor, Harvard University, Boston, Mass.

† We are indebted to Dr. John T. Tripp of the Laboratory of Biologics Control, NMI, for the use of this apparatus and for generous donations of various plasma samples.

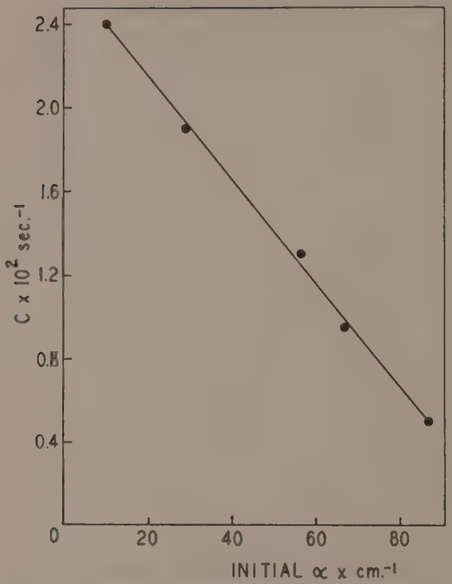

FIG. 1. Relation between rate of inactivation of siderophilin and absorption coefficient of the test solution.

taining solution and the decrease in its TIBC. The C values listed are the first order rate constants calculated on the basis that the effective radiation intensity was constant. It will be observed, however, that the absolute values of C are different for each of the several siderophilin solutions tested and that throughout the period of exposure of any one solution, the C values changed. Hence, these values are not true rate constants.

The magnitude of the C value for a given test solution is dependent upon the absorption coefficient (*α*) of that solution—the higher the *α* value the lower the C value. The relationship between C and *α* is illustrated in Fig. 1 in which the inactivation rates are plotted for solutions containing 2-3 mg siderophilin per ml and exhibiting a range of initial *α* values from 10.1 to 87. The solutions of intermediate *α* values were prepared by the appropriate dilutions of ACD plasma with the fraction IV-7 solution. The apparent rate constants were obtained by determining the time required for 50% inactivation (t½) from a plot of $\log \frac{\text{TIBC at t} = \text{o}}{\text{TIBC at t}}$ versus time t (seconds) and substituting the derived t½ in $C = \frac{0.693}{t\frac{1}{2}}$. It was necessary to extrapolate the linear plot to obtain the t½ in cases where deviation from linearity occurred before this point was reached. The progressively decreasing rate of inactivation of siderophilin in the test solutions of increasing *α* value can be ascribed to the enhanced "internal screening" (5) of the effective radiation by the non-siderophilin plasma proteins.

The fact that the value of C did not remain constant during the period of irradiation of any one solution suggested that its initial *α* value changed during the exposure period. Experiments to elucidate this point showed that the *α* value of a given test solution did increase linearly for at least the first 50 seconds of exposure, *e.g.*, a plasma sample of initial *α* value of 67 increased 0.125 *α* unit/second while a fraction IV-7 solution with an initial *α* of 10.1 increased 0.11 *α* unit/second. For longer periods than 50 seconds, additional factors probably played a role in determining the amount of radiation absorbed. One such factor was the progressive formation of a visible surface film of coagulated protein clearly evident after 150 seconds exposure.

Since approximately one-third of the siderophilin present in normal plasma is iron saturated, experiments were performed to determine whether there was any difference in sensitivity to UVR of the iron-saturated and iron-free siderophilin in blood plasma. Table II illustrates the UV inactivation of siderophilin-enriched plasma solutions, 25 and 100% iron-saturated. The inactivation of the iron-saturated sample was measured by determination of the iron dissociated from the complex under conditions comparable to those used for determining the unsaturated iron-binding capacity (UIBC). Subject to the limitations of the methods employed, the inactivation rates of the iron-free and iron-saturated siderophilin species were the same.

Summary. 1. Ultraviolet irradiation of ACD plasma and of plasma fraction IV-7 solutions inactivates the siderophilin component as measured by the loss of its specific iron-binding capacity. 2. The initial inactivation

TABLE II. Effect of UVR on Iron-Saturated and Iron-Unsaturated Plasma Siderophilin.

	Iron-saturated—100%			Iron-free— 74.4% Iron-saturated—25.6%		
UVR time (sec)	TIBC, μg Fe/ml	% TIBC	$C \times 10^2 \times sec^{-1}$	TIBC, μg Fe/ml	% TIBC	$C \times 10^2 \times sec^{-1}$
0	3.94	100		3.94	100	
25	2.99	75.9	1.10	2.93	74.5	1.18
50	2.61	66.3	.82	2.76	70	.71
100	2.16	54.8	.60	2.06	52.3	.65
150	1.82	46.2	.52	1.93	49.1	.47

rates, whose magnitude was correlated with the absorption coefficients of the irradiated siderophilin solutions, followed essentially first order kinetics. 3. The UV inactivation rates of iron-free and iron-saturated siderophilin were found to be the same.

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Effect of New Adrenal Steroids on Electrolyte and Water Excretion. (22173)

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Recent clinical work has indicated that the Δ^1-analogues of hydrocortisone and cortisone, namely prednisolone and prednisone, are in certain respects more potent than the parent compounds and differ from them qualitatively in that they cause little or no sodium and water retention at therapeutically effective doses(Bunim, *et al.*, 1, 2; Spies, *et al.*, 3; and others). Pechet and Bartter(4) found that in Addisonian patients prednisone would cause sodium loss whereas cortisone caused sodium retention. This action was correlated with a marked ability of the Δ^1-compounds to increase the glomerular filtration rate. In animal studies, Perlman and Tolksdorf(5) found the Δ^1-steroids more potent than their natural counterparts in causing liver glycogen deposition, eosinopenia and life-maintenance, but their effects on electrolyte excretion were unchanged. In tests designed particularly to measure sodium-retaining potency, Stafford, *et al.*(6) found prednisolone to have "very slight" activity.

It is well known that adrenal cortical hormones are necessary for the maintenance of a normal rate of water diuresis and that deficiencies in this respect are repaired effectively by hydrocortisone and cortisone, but weakly, if at all, by desoxycorticosterone. We considered it of interest to determine, therefore, the relative activity of hydrocortisone, desoxycorticosterone (DC), prednisone* and prednisolone in this test. Excretion of sodium and potassium was measured also in the water-loaded test animals. Under the experimental conditions used, hydrocortisone and cortisone are known to increase sodium and potassium as well as water excretion.

Methods. Fasted male rats weighing approximately 200 g were tested 18 hours after adrenalectomy. At the beginning of an ex-

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* Prednisone (Meticorten) and prednisolone (Meticortelone) were generously supplied to us by Schering Corp.

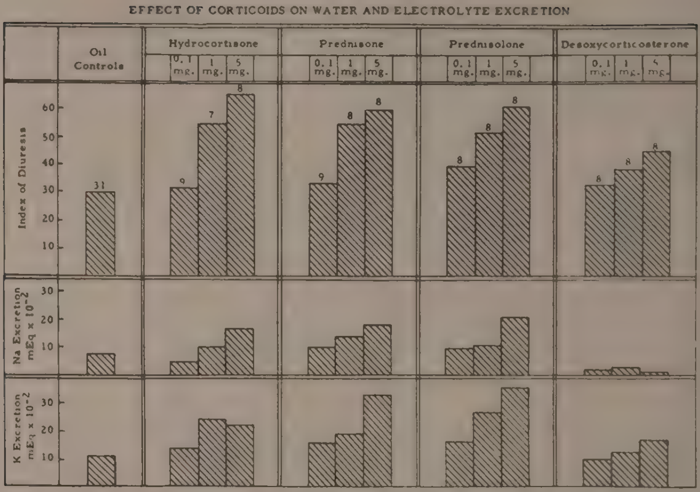

FIG. 1. Effect of various corticoids on water, sodium and potassium excretion over a 5-hr period in water-loaded adrenalectomized rats. Numbers above columns indicate number of animals on which mean values are based. "Index of Diuresis" represents in cm^2 the area under cumulative water excretion curves; increasing numbers represent increasing rates of water excretion.

periment, they were given 2 doses of distilled water (3 ml/100 cm^2 — ref. 7) an hour apart by stomach tube. The rate of water excretion was followed at half-hour intervals for 5 hours, starting from the first water load, and the total amounts of sodium and potassium excreted in that time were determined by flame photometry.

To express the effect of treatment on water excretion as one figure, the method of Krieger and Kilvington(8), as used by Ham and Landis(9), was employed: for each rat the rate of cumulative water excretion, expressed as per cent total load given, was plotted for the 5-hour period. The scale of the graph was such that 1 cm on the ordinate equaled 10% excretion of the total water load and 1 cm on the abscissa equaled 30 minutes time. The area under each curve was measured with a planimeter and the region encompassed in sq. cm designated the *Index of Diuresis*. Steroids were administered in oil, either in solution (small doses) or partial suspension (large doses) in amounts of 0.1 mg to 5 mg/rat. One-half of the total dose was injected subcutaneously at the time of adrenalectomy and one-half 30 minutes before the first water load. Control rats received sesame oil.

The significance of possible differences between the different treatments was tested primarily by analyses of variance (Snedecor, 10).[†] Since the nature of the results is in most respects self-evident from the graphs, statistical details are not included here.

Results. 1. *Water Excretion.* All of the steroids tested enhanced the sluggish rate of diuresis which characterizes adrenal insufficient animals and man (Fig. 1). In this respect no clearly significant differences were observed between the action of hydrocortisone, prednisone and prednisolone at any dose level. All 4 of the compounds tested gave dose-response curves with essentially parallel slopes. The response to DC, however, was definitely weaker ($P = <.01$) than any of the other compounds. None of the steroids was clearly effective at 100 μg dose levels.

2. *Potassium Excretion.* All 4 compounds increased potassium excretion (Fig. 1). Again DC was the weakest of the 4 and consistently active only at 5 mg doses.

3. *Sodium Excretion.* The only qualitative difference observed between the 4 compounds

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[†] The authors are indebted to Mr. James Webster for statistical analysis of these data.

was that DC caused sodium retention and the other 3 steroids enhanced sodium excretion (Fig. 1). The 3 natriuretic compounds were equi-potent.

Discussion. It has been repeatedly demonstrated that the action of adrenal cortical steroids on water and electrolyte excretion can be altered or reversed depending upon a number of variables, some of which are undefined. For instance, hydrocortisone may cause either sodium retention or sodium diuresis when used in different rat tests specifically designed to detect and measure sodium-retaining activity. In other words, the response to the steroid is determined in part by intrinsic properties of the steroid itself, but equally or more so by the physiological environment with which it is provided. The present results and those of Perlman and Tolksdorf(5) suggest that those alterations in sodium-retaining properties of certain steroids resulting from the presence of a 1-2 double bond do not alter their ability to enhance sodium excretion under conditions where such activity might be expected.‡

From all of the work now available it is apparent that the Δ^1-steroids are, relative to their parent compounds, weaker in certain respects (*e.g.*, sodium retention in clinical use), stronger in several properties (anti-inflammatory effects, etc.) and of unchanged potency in others (water diuresis, etc.).

Summary. The effects of prednisone, prednisolone, hydrocortisone and desoxycorticosterone on excretion of water, sodium, and potassium, were compared in water-loaded adrenalectomized rats. All steroids stimulated water diuresis and potassium excretion. Hydrocortisone, prednisone and prednisolone were equi-potent; desoxycorticosterone was weaker. Hydrocortisone, prednisone and prednisolone stimulated sodium excretion at all doses having any effect and were again approximately equi-potent. Desoxycorticosterone had the opposite effect and caused sodium retention. The relative inability of the Δ^1-steroids to cause sodium retention under conditions of clinical use is, therefore, not associated with any comparable inability to cause natriuresis under appropriate circumstances.

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‡ In addition, the authors have found (unpublished) that hydrocortisone, prednisone and prednisolone have about equal potency in antagonizing the antidiuretic effect and enhancing the natriuretic effect of exogenous vasopressin.

Progestational Activity of Halogenated Corticosteroids and Related Compounds in Rabbit and Monkey.* (22174)

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Fried(1) has reviewed the biological effects of the 9 alpha halo derivatives of hydrocortisone, cortisone, 11-beta-hydroxyprogesterone and other related steroids. The high mineralocorticoid and glucocorticoid activity shown by several of these compounds in experiments with rats and dogs were described. Results of the present investigations demonstrate progestational activity of several of the 9 alpha halo steroids in both the rabbit and monkey.

Materials and methods. Clauberg rabbit test. Female New Zealand white rabbits weighing 600-800 g at the beginning of treatment were used in all experiments. Ten μg of estradiol 17-beta were given daily subcutaneously in 0.25 ml sesame oil solution for 6 days. Beginning on the 7th day, the test compounds were given subcutaneously daily for 5 days. The animals were killed with chloroform 20 to 24 hours after the last injection. Body, uterus and adrenal weights were recorded. Uteri and adrenals were fixed in Bouin's solution. Sections were stained with hematoxylin and eosin. Progestational proliferation of the endometrium was measured according to the standard scale of McPhail (5). *Inhibition of estrogen withdrawal bleeding in the monkey.* Bilaterally ovariectomized female monkeys (*Macacus rhesus*) weighing 3.5-5.5 kg were given daily subcutaneous injections of 10 μg estradiol 17-beta in 0.25 ml sesame oil solution for 20 days. Subcutaneous injections of the test compound were begun on the 21st day. Daily vaginal smears were made with normal saline. Treatment was discontinued either with the onset of menstrual bleeding or when the experiment was terminated in order to obtain samples of the endometrium. With the exception of progesterone and desoxycorticosterone acetate (in solution), all compounds were administered as suspensions in sesame oil.

Results. It is well to keep in mind the quantitative variability of the bioassay method when the potency of compounds is compared by means of the Clauberg rabbit assay. We have chosen a total dose of 0.5 mg progesterone as the reference level because this amount will produce full progestational proliferation of the endometrium in more than 75% of the rabbits receiving this dose whereas 0.25 mg progesterone gives a maximum progestational response in 28% of animals on this lower dose. These percentages were derived from a total of 61 rabbits on the 0.5 mg dose and 22 rabbits on the 0.25 mg dose.

Data from several experiments are presented in Table I. It will be noted that at a total dose of 5 mg, the activities of 9 alpha-fluoro-hydrocortisone acetate, 9 alpha-chloro-hydrocortisone acetate and desoxycorticosterone acetate are comparable whereas hydrocortisone acetate is inactive even at a level of 25 mg. At a total dose of 15 mg, both the 9 alpha-fluoro- and 9-alpha-chloro derivatives of hydrocortisone produce a response comparable to that of 0.5 mg of progesterone (full proliferation). This would indicate an activity for these halo compounds of about 3% that of progesterone.

Delta-1-9-alpha-fluorohydrocortisone acetate was also assayed by the Clauberg method. This compound gave minimal progestational responses both at total doses of 10 mg and 20 mg.

Table I includes results on progestational proliferation produced in response to treatment with 9-alpha-fluoro-11-beta-hydroxyprogesterone. A 1.0 mg total dose of this halogenated compound possesses low grade activity and at the 3.0 mg level there is a moderate response. At 5.0 mg a maximal response was educed. An estimated potency of 5 to 10% that of progesterone could be made for this

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* The 9 alpha-halo steroids used in this study were supplied through the courtesy of Dr. J. Fried of the Squibb Institute for Medical Research. The delta-1 steroids were supplied through the courtesy of Dr. Marvin Kuizenga of the Upjohn Co. and Dr. Preston Perlman of the Schering Corp.

TABLE I. Progestational Activity of Halogenated Steroids in the Clauberg Assay.

	Progestational proliferation					
Total dose (mg)	9 alpha-chloro F acetate	9 alpha-fluoro F acetate	Cpd. F acetate	DOC acetate	9 alpha-fluoro 11-beta-hydroxy-progesterone	Progesterone
25			0, 0, 0			
15		4, 3, 4				
	4, 4, 4*	4, 4, 4				
10		3, 2, 2				
	3, 4, 4, 1, 2	3, 3, 2	0, 0, 0	4, 4, 3	4, 4	
5	2, 2, 1	2, 1, 1				
	2, 1, 1	1, 1, 1	0, 0, 0	2, 1, 1	4, 4	
3.0					3, 2	
2.5	0, 0, 0	0, 0, 0				
	0, 0, 0		0, 0, 0	2, 2, 1		
1.0	0, 0, 0	0, 0, 0			2, 2, 1	
.5						4, 4, 4
	0, 0, 0					3, 4
.25						4, 4, 3, 3, 3

* Each number represents degree of progestational proliferation for one animal.

halo steroid. Neither delta 1-hydrocortisone nor delta 1-cortisone showed progestational activity when tested at a total dose of 20 mg using 3 animals for assay of each compound. Cortisone acetate was inactive as a progestogen at a total dose of 50 mg (3 rabbits).

Gross examination of animals receiving the 10 and 15 mg doses of fluoro- and chlorohydrocortisone and the 25 mg dose of hydrocortisone revealed ascites and liver enlargement. Adrenal weights were consistently lower in these animals as compared with those on progesterone treatment.

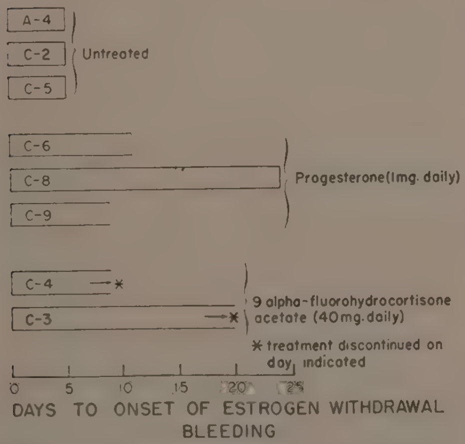

FIG. 1. Progestational activity of 9 alpha-fluorohydrocortisone acetate in the monkey.

Results obtained from experiments with estrogen-primed castrate female rhesus monkeys are shown in Fig. 1. Three untreated monkeys began estrogen withdrawal bleeding on the fifth day following the last injection of estradiol. The onset of bleeding was delayed from 9 to 24 days in 3 monkeys receiving 1.0 mg progesterone daily. Of 2 monkeys treated with a daily dose of 40 mg of 9 alpha-fluorohydrocortisone acetate, one continued for 9 and the other for 18 days without withdrawal bleeding. Treatment of these animals was discontinued to permit uterine biopsies. Histological examination of the biopsies presented a clearly defined progestational endometrium. In another animal, a daily dose of 20 mg of 9 alpha-fluoro-hydrocortisone acetate failed to delay estrogen withdrawal bleeding.

Our results demonstrated progestational activity of 9 alpha-chlorohydrocortisone, 9 alpha-fluorohydrocortisone and 9 alpha-fluoro-11-beta-hydroxyprogesterone in the rabbit. Hydrocortisone was shown to be inactive even at high levels. Delay of estrogen withdrawal bleeding in the monkey further demonstrates the progestational action of 9 alpha-fluorohydrocortisone. The lack of toxicity and any unusual side effects of this halogenated corticosteroid in the monkey is in marked contrast with effects noted in the rabbit.

Discussion. Byrnes and Shipley(2) reported a lack of progestational activity for 11 beta-hydroxyprogesterone at a dose of 5 mg in the Corner-Allen assay. It is evident that the introduction of either a chlorine or a fluorine atom in the 9 alpha position imparts progestational activity in hydrocortisone and 11-beta hydroxyprogesterone both of which were progestationally inactive at comparable doses.[†] Junckmann(3) has reported that 17 alpha-hydroxyprogesterone, slightly active as a progestational compound, becomes active upon esterification. Huggins and Jensen(4) have recently described the potent depressant activity of 9 alpha-fluorohydrocortisone and 9 alpha-11-beta-hydroxyprogesterone on estrone-induced uterine growth in the hypophysectomized rat. Hydrocortisone and cortisone were considered to be weak inhibitors in the same test.

Summary. Progestational activity of 9 alpha-chlorohydrocortisone acetate, 9 alpha-fluorohydrocortisone acetate and 9 alpha-fluoro-11-beta-hydroxyprogesterone has been demonstrated using progestational proliferation of the rabbit endometrium (Clauberg test) as index of activity. Hydrocortisone acetate and cortisone acetate show a lack of activity in the same test. Progestational activity of 9 alpha-fluorohydrocortisone is further demonstrated by prevention of estrogen withdrawal bleeding and characteristic histological changes in the endometrium of the ovariectomized rhesus monkey pre-treated with estrogen.

[†] However, 11-beta-hydroxy progesterone has one-eighth the activity of 9 alpha-fluoro-11-beta-hydroxy progesterone in the Clauberg Test.

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Metabolism of the Lanthanons in the Rat.* (22175)

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For many years the behavior of the lanthanons in biological systems was regarded as a scientific curiosity. The name "lanthanon" has been suggested(1) for the group of elements of atomic numbers 57 to 71, lanthanum through lutetium. Until recently the commercial use of these elements was limited and their chemistry relatively obscure. With the development of the chain-reacting pile, and the discovery that certain light members of this group were produced in relatively large quantities in the fission process, knowledge of their metabolism[†] and radiotoxicity assumed greater importance. Certain members of this group can be reduced to the divalent state and others oxidized to the quadrivalent state; the principal valence state of the group as a whole, however, is plus three. Although the actual radius of an ion of a particular element depends principally upon its valency, the radii of the tervalent ions of the lanthanons show an interesting decrease with increasing atomic number. This phenomenon

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* This work was performed under auspices of Atomic Energy Commission.

[†] There has been much dissension concerning the use of the word "metabolism" for elements not intimately involved in biological processes; this terminology, however, is in general use in tracer work of the type presented here.

has been called the "lanthanide contraction." (2) As the ionic radius decreases, the basicity of the lanthanons decreases and their compounds become more soluble, which may, to some extent, account for the differences in distribution of the light and heavy lanthanons described here. A complete review of the history, occurrence, applications, and chemistry of the lanthanons has been published by Vickery(3). The relationship of the lanthanons to biological systems was studied almost as soon as the first members of the group were isolated. A complete bibliography to 1935 on the biological occurrence, metabolism, and toxicity of the lanthanons has been prepared (4). In many biological specimens traces of cerium and lanthanum were found. A survey of the experimental exposure of living systems to lanthanons indicates that in vertebrates the toxic effects following parenteral injection were probably due to the formation of insoluble phosphates and carbonates, which disturb the hydrogen ion concentration of the organism. The observation was frequently made that the lanthanons were nontoxic (even in large amounts) when given orally. This was probably because they are poorly absorbed from the gastrointestinal tract. Under the auspices of the Manhattan Project, tracer studies were performed in this laboratory(5,6) with some of the lighter members of the lanthanon series that are produced in nuclear fission. Some of these were carrier-free,[‡] and others were administered with considerable amounts of stable isotope carrier, making direct comparisons difficult.

The studies reported here were designed to correct some of the defects of the earlier work and to expand the data to include all the lanthanons on an uniform and systematic basis. The recent availability of extremely pure specimens of the rarer lanthanons, and the construction of the high-neutron-flux Materials Testing Reactor, made possible the production of the necessary quantities of previously unobtainable radioisotopes of high specific activity. The age, sex, and the strain of the rats, and the care given them, were uniform throughout. The size of the groups was increased. The radioisotopes were administered as citrate complexes, which increased the speed of absorption from the site of injection.

Methods. Preparation of Radioisotopes. The radioisotopes of cerium, europium, and promethium were obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee. The praseodymium data are reprinted with the kind permission of Josephine Crowley-Ellis(7). Radioactive thulium and terbium were provided by Drs. Glenn T. Seaborg and Stanley Thompson of the University of California Radiation Laboratory. Dr. Frank Spedding of the University of Iowa, Ames, Iowa, very kindly supplied 99.9 to 99.95% spectroscopically pure oxides of the remaining lanthanons from which radioisotopes were prepared by the (n,γ)[§] reaction. Weighed portions of the oxides were diluted in 6 *N* HCl. Aliquots were transferred to clean quartz ampules. The ampules containing the lanthanon solutions were dried overnight in an oven at 105°C and heated at 200°C for a few minutes to expel all traces of water. They were then sealed and sent to Arco, Idaho, where they were placed in the Materials Testing Reactor in the region of maximum neutron flux. After bombardment, the materials were flown back to this laboratory. The ampules were placed in heavy plastic envelopes and crushed. The crushed ampule was transferred to a beaker and washed several times with 6 *N* HCl or until a survey meter showed that no more activity could be removed from the quartz. Ten milligrams of NaCl were added to the solution, which was evaporated to incipient dryness. Sodium citrate solution, 30 mg/ml, was added, the amount varying with the quantity of activity present, and the pH was adjusted to neutral with 9 *N* NaOH. The latter procedure—evaporating with salt and redissolving—was also followed with the isotopes obtained from

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[‡] A carrier-free preparation is one in which all the atoms present of a particular element are radioactive. The specific activity of such a preparation is by definition, $\frac{\text{N active}}{\text{N total}} = 1$.

[§] Dy^{166} was prepared by a second-order reaction, $Dy^{164}(n,\gamma)$, $Dy^{165}(n,\gamma)$, Dy^{166}.

TABLE I. Summary of Time Intervals Investigated and the Amounts of Rare Earth, and Complexing Agent Administered per Rat.

Isotope	Time interval (days)	Dose (μc)	Carrier (μg)	Sodium citrate (mg)
La^{140}	1, 4	29, 58*	1.2, 2.4	6, 12
Ce^{144}	1, 4, 64, 256	20–40†	c.f.‡	3–6
Pr^{143}	1, 4, 15, 32	180	c.f.	2
Nd^{147}	1, 4, 16	2.3–4.6	1.1–3.7	2.7–5.6
Pm^{147}	1, 4, 64, 238	10–70	c.f.	3–6
Sm^{153}	1, 4	30, 75	0.3, 0.6	3, 7.5
$Eu^{152,154}$	1, 4, 64, 256	1.8–2.6	5.2–7.8	5.7–8.6
Gd^{159}	1, 4	3.8, 20	2.0, 10.8	2.3, 12
Tb^{160}	1, 4, 64, 256	3.0–12.0	3.0–12.0	2.8–11.2
Dy^{166}	1, 4	4.3, 11.0	0.1, 0.3	3.0, 7.5
Ho^{166}	1, 4	14, 84	0.02, 0.12	1.3, 7.8
Er^{169}	1, 4, 16	2.4–4.8	1.0–3.8	2.8–5.6
Tm^{170}	1, 4, 64, 256	3–12	0.08–0.3	3–12
Yb^{175}	1, 4, 8	14–57	0.4–1.1	2.5–7.4
Lu^{177}	1, 4, 16	7.3–29	0.5–1.9	4.8–7.2

* One- and four-day dosages respectively.
† Dosages graded for time intervals involved.
‡ c.f. = carrier-free.

other sources. The radioactive purity of all the preparations, and the identity of the isotopes were determined by the measurement of the half life, the beta-ray absorption in aluminum, and the gamma-ray absorption in lead. The specific activities were estimated from the weight of the bombarded sample and the total volume of the final citrate solution. The half lives, beta energies, and the presence or absence of gamma rays of the isotopes employed are listed in Hollander *et al.*(8).

Biological Procedures. The animals used in these tracer studies were female Sprague-Dawley rats that were from four to six months of age (200-250 g) when injected. The animals were maintained on tap water and a pelleted stock diet developed by the University of California Institute of Experimental Biology, "Diet 14," for at least two weeks prior to isotope administration and until sacrifice. While the animals were under light ether anesthesia, isotopes of all the lanthanons were administered intramuscularly in the left hind leg and Ce^{144}, $Eu^{152,154}$, Tb^{160}, and Tm^{170} were also given orally by stomach tube. In no case did the volume of solution administered exceed 1 ml. The time intervals investigated, the dosage of each radioisotope in microcuries per rat, the amount of stable lanthanon per rat, and the amount of sodium citrate administered per rat are shown in Table I. Moderate amounts of high-specific activity preparations were used in order to avoid uncertainties introduced by either radiotoxicity or chemical toxicity. The largest amount of radioactivity administered, 0.5 $\mu c/g$ of body weight, was felt to be well below radiotoxic level(9). The amount of complexing agent used was roughly adjusted to the quantity of carrier present. The actual dosage for any given experiment, both in microcuries and micrograms, depended on the best balance obtainable among the following factors: specific activity, half life, counting accuracy desired, and in most cases, beta-particle energy. In the intramuscular studies at least 5 rats were injected for each time interval.‖ In the oral studies, groups of three animals were used. A standard was prepared for each experiment; at the time of injection, an amount of the radioactive solution equal to that administered to each animal was diluted to 100 ml in 2 N HNO_3. Following administration of isotope, groups of 2 or 3 animals were placed in metabolism cages which were designed to provide adequate separation of urine and feces with a minimum of handling. Excretions were collected daily for the first 4 days after injection and twice weekly thereafter. In the intramuscular studies animals were sacrificed at intervals of one and 4 days after injection in all the experiments, and when the half life permitted, at longer time intervals ranging from 8 to 256 days. In the oral studies the animals were sacrificed at four days. Under chloroform anesthesia, a blood sample was withdrawn by heart puncture, after which the animals were returned to the chloroform for sacrifice. The left hind leg (the site of injection) was removed at the pelvic girdle, and the foot was cut off for assay with the carcass. The remaining carcass was then skinned, and the following tissues and organs were dissected and weighed: pelt, spleen, liver, kidneys, gastrointestinal tract, gastrointestinal contents, the muscle from the right hind leg, and the femur, fibula, and tibia from the right hind leg.

‖ An outbreak of pneumonia in the colony reduced the number of rats to four in the 256-day $Eu^{152,154}$, and Tb^{160} experiments.

Preparation of Samples for Beta Assay. The blood, spleen, kidneys, muscle, and gastrointestinal tract (small samples) were placed in individual porcelain ashing capsules, and the liver, bone, skin, gastrointestinal contents, left leg, carcass and excreta (large samples) were placed individually into beakers. All tissues were dried for 24 hours at 100°C and ashed for 24 hours at 500°C. The ash from the small samples was spread evenly by dissolving in 2 N HNO_3 and redrying. The soft-tissue ash in the carcass was removed from the skeleton by rinsing with distilled water and filtering through gauze. The skeletal ash was dried and weighed. The large samples, including skeleton, were dissolved in 2 N HNO_3, and aliquots were transferred to weighed porcelain capsules and dried. To correct for self-absorption of the beta particles in the mass of the sample, dilution curves were prepared for each isotope according to the methods previously described(10). The weights of the large samples were determined by difference. The weights of the small samples were estimated on the basis of 1.25% ash in soft tissues. An aliquot of the standard was assayed whenever samples were counted to eliminate corrections for decay and fluctuations in counter efficiency. A self-quenching Geiger-Muller counter was employed for the beta particle assays, and all samples were counted for a sufficiently long time to reduce the error of measurement to ± 5%(11).

Preparation of Samples for Gamma Counting. Gamma rays were present in sufficient intensity for accurate measurement of La^{140}, Ce^{144}, Sm^{153}, and Yb^{175}. The procedures employed for preparation of the skeleton and excreta samples were the same as described in the previous section. Other tissues and organs were placed in tin bottle caps. The samples were assayed wet with a TlI-NaI scintillating crystal counter of the type described by Jenkins(12).

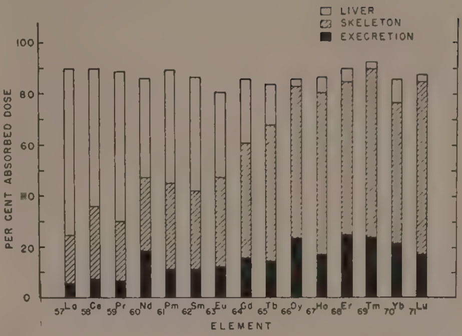

FIG. 1. Summary of the excretion, skeletal deposition, and liver deposition of the lanthanons one day after intramuscular administration. The difference between the end of each bar and 100% represents the % of each isotope in the remainder of the animal.

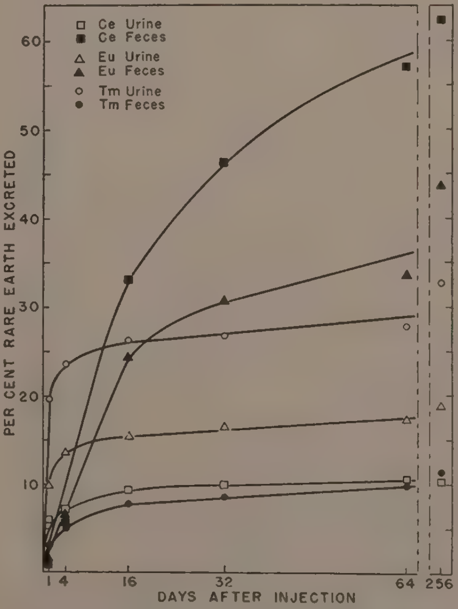

FIG. 2. The cumulative excretion of Ce^{144}, $Eu^{152,154}$, and Tm^{170} in urine and feces. Values are corrected for deviation of recovery from 100% and are expressed as % of absorbed dose. The one-day points represent eight determinations, the four-day points six determinations, the 16-, 32- and 64-day points four determinations, and the 256-day points two determinations.

Calculations. The total amounts of radioisotopes present in blood and muscle were calculated on the basis that these two tissues represent 7 and 45% of the body weight respectively. The lanthanon concentration in bone was calculated from the ash content of mature rat bone, 36.6 ± 0.7%. The amount

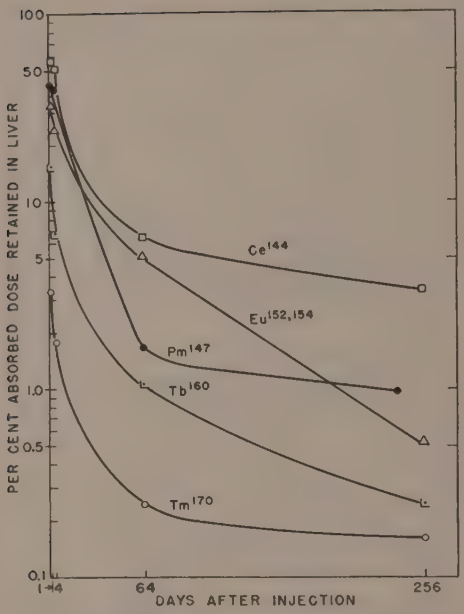

FIG. 3. The liver retention of Ce^{144}, Pm^{147}, $Eu^{152,154}$, Tb^{160}, and Tm^{170}. Each point represents five animals.

of each isotope remaining at the site of injection was calculated by subtracting the activity present in the muscle and bone samples from that found in the whole left leg. The residual per cent of dose, which has been designated "balance" consisted of the thoracic organs; reproductive organs; cartilage; glandular, lymphoid, connective and nervous tissue; fat; and blood vessels. All data are expressed as concentration and as the per cent of the absorbed dose found in the whole specimens, and are corrected for deviation of recovery from 100%.

Results. The trend of the results of the tracer studies with the fifteen lanthanons is represented in Fig. 1, which shows the per cent of administered dose in liver, skeleton and excreta one day after lanthanon administration. Fig. 2 shows the cumulative excretion of three representative lanthanons. The retention of five representative lanthanons in the liver and skeleton are shown in Figs. 3 and 4.

The experimental recoveries of the injected material ranged from 86% for the 256-day Tm^{170} to 121% for the 4-day Lu^{177}. Recoveries below 95% or greater than 105% were exceptional, however, indicating that on the whole the data are reliable.

Absorption. Absorption from the intramuscular injection site was, for the most part, fairly complete. In 11 of 15 experiments, the amount of isotope unabsorbed four days after injection was less than 6.5%. Three of the four exceptions, $Eu^{152,154}$, Gd^{159}, and Tb^{160} (15% unabsorbed at 4 days), were administered with 8 to 10 μg of carrier, the largest quantities employed. The ratio of micrograms carrier to milligrams sodium citrate was 0.9, also considerably higher than in most of the experiments. In the La^{140} studies (22.5% unabsorbed at 4 days), the ratio of carrier to complexing agent was 0.2 μg/mg. The insolubility of the compounds of lanthanum at physiological pH's is probably a major factor in its difficult absorption. The rate of absorption of the lanthanons from the intramuscular injection site seems to depend upon the solubility of their compounds, the amount

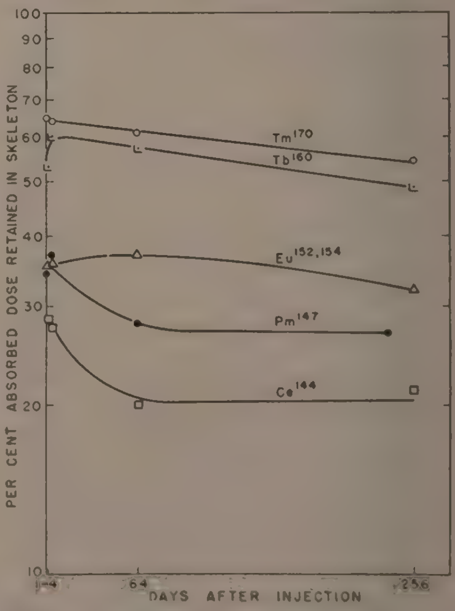

FIG. 4. The skeletal retention of Ce^{144}, Pm^{147}, $Eu^{152,154}$, Tb^{160}, and Tm^{170}. Each point represents five animals.

of stable carrier administered and, to some extent, upon the ratio of the quantity of carrier to the quantity of sodium citrate administered. Absorption of Ce^{144}, $Eu^{152,154}$, Tb^{160}, and Tm^{170} from the gastrointestinal tract was less than 0.1% of the administered dose. Because of the great mass of the data, only significant trends will be discussed.[¶]

Spleen. The concentration of the lanthanons in the spleen one day after administration was less than 0.5% per g of wet tissue. When these elements are administered by vein as halides or nitrates, high concentrations are found in the spleen as well as in other components of the reticulo-endothelial system. Laszlo *et al.*(13) concluded that they were dealing, to a large extent, with radiocolloids. Bruner *et al.*(14) in studies with radiogallium (principal valence plus three) reached similar conclusions. The intramuscular route was chosen in the present experiments in order to minimize the formation of insoluble lanthanon colloids in the blood stream. The low spleen concentrations demonstrated that this aim was largely achieved; that these data represent the metabolism of these elements in non-colloidal form.

Blood, Muscle, and Skin. The level of these elements in the blood at one day never exceeded 0.02% per ml, and there was a steady decrease to unmeasurable levels thereafter. The initial muscle concentration of the lanthanons was the same as for blood, but declined at a somewhat slower rate. The skin concentrations throughout the series, at the early time intervals, were about three to four times as great as those found for muscle and blood, subsequently diminishing at about the same rate as did the concentrations in muscle. It is possible that at the early time intervals there may have been some contamination of the skin by the urine. This would apply particularly to the heavy members of the series, for which the urine is the main route of excretion.

Gastrointestinal Tract. The gastrointestinal tract generally showed a concentration about ten times that of blood and muscle at the earlier time intervals. Two to eight months after administration the gastrointestinal concentrations had dropped to low but still measurable levels in the lighter half of the series, for which the gastrointestinal tract is the chief excretion route, and to unmeasurable levels in the latter half of the series. Retained fecal matter, particularly in the cecum, may have contributed to the initial high concentrations in the gastrointestinal tract, because the contents were merely squeezed from it.

Balance. The soft-tissue balance, consisting of the thoracic organs and other tissues previously enumerated, contained less than 6% of the administered dose throughout the series, indicating that the values employed for total blood and total muscle were satisfactory, and that the separation of the soft-tissue ash from skeletal ash was relatively good.

Kidney. Initially the concentration of the light lanthanons in the kidney was about 1.6% per g, a much greater concentration than was found for any other soft tissue except liver. The kidney concentrated more of the heavy lanthanons than did any other soft tissue, about 0.9% per g. Eight months after administration the concentration in the kidney had, in all cases, decreased to low levels. There was no apparent correlation between either the 1-day or 8-month kidney concentrations and the quantity of the isotope excreted in the urine.

Excretion. The lanthanons are excreted both by the kidney and by the liver via the gastrointestinal tract. The relative importance of either excretion route for any given element of the series is dependent upon its position in the series. More than 50% of the administered dose of the lighter lanthanons is accumulated by the liver, and it is thereafter rapidly excreted in the feces. The heavier lanthanons are excreted primarily by the kidney. Those lanthanons in the middle of the series are excreted by both routes and to approximately the same extent. The cumulative excretion in both urine and feces is shown in Fig. 2 for Ce^{144}, $Eu^{152,154}$, and Tm^{170}. Very little of any of the lanthanons was excreted in the urine after the first week postinjection, although there was a slight tendency towards continued urinary excretion of the heavier members of the series. Most of the fecal excretion of Tm^{170}, and presumably of the other heavy lanthanons, occurred in the first two weeks after administration. Fecal excretion of the light and medium weight lanthanons occurred throughout the entire eight-month investigation, but at a slower rate after the first two weeks.

[¶] The tabular data are available from the authors upon request.

Liver. The major deposition site of the lighter members of the series is the liver. At the heavy end of the series the liver is a secondary deposition site and contains less than 5% of the initially administered radioisotopes. The retention of five representative members of the lanthanons in the liver is shown in Fig. 3. All the lanthanons are excreted from the liver, with half times ranging from 10 to 20 days, so that two months after injection less than 10% of that initially deposited remains.

Skeleton. The most important deposition site for all the members of this group is the skeleton. It is the primary target organ of the heavier lanthanons, and although initially it is apparently a secondary deposition site for the lighter members of the group, it becomes increasingly important because of their extended retention at this site. The skeletal retention of five representative lanthanons is shown in Fig. 4. The retention curves obtained for Ce^{144} and Pm^{147} (lighter lanthanons) show two components. One-third of these isotopes initially deposited in bone is somewhat labile with a half time of about fifteen days, and the remaining two-thirds is apparently firmly fixed. The curve obtained for $Eu^{152,\ 154}$ is unique in that it is convex. This presumably is due to the delayed deposition in the skeleton of $Eu^{152,154}$, which was only gradually absorbed from the site of injection. The skeletal half time for $Eu^{152,154}$, based on the best straight line through all the points is about 2.5 years. The retention curves for Tb^{160} and Tm^{170} were straight lines for which the half-time values are about 2.8 years.

Discussion. The data shown here establish definite differences in excretion patterns and deposition sites between the light and heavy lanthanons; however these differences seem to be more a matter of degree than of kind. According to their behavior in the mammalian organism, the lanthanons can be divided into the same three groups familiar to modern lanthanon chemists: (3) the light lanthanons —lanthanum through samarium; the transition group—europium, gadolinium and terbium; and the heavy lanthanons—dysprosium through lutetium.

Radioautographs of long bones(5) and of costochondral junction(15) demonstrated that in the normal rat Ce^{144} and Pm^{147} (light lanthanons) are deposited on the endosteal and periosteal surfaces and in the vicinity of the small blood vessels in the compact bone. Copp *et al.*(16) compared the skeletal distribution in normal adult, normal young, and rachitic rats of the alkaline earths, Ce^{144} and Y^{88}. On the basis of chemical properties and metabolic behavior(16), Y^{88} can be grouped with the heavy lanthanons. They found that the skeletal deposition of Ce^{144} was moderately increased in normal young over normal adults, and strikingly increased in rachitic rats. The amount of Y^{88} deposited in the skeleton (about 70% of the administered dose) was unaffected by either rapid skeletal growth or phosphorous deficiency. Radioautographs of rachitic bone demonstrated that both Ce^{144} and Y^{88} were deposited almost entirely superficially in the shaft and in the uncalcified osteod matrix below the epiphysis. All these findings strongly suggest that the lanthanons have a specific affinity for the bone protein even in the absence of bone salt.

The increased skeletal deposition of the heavier members of the lanthanon series and the difference in skeletal retention curves for the light and heavy lanthanons suggest that these two groups may be handled somewhat differently in the skeleton. It is likely that these differences are determined by the type or strength of the chemical combination of the lanthanons with the bone protein. Ketelle and Boyd(17) have made an interesting presentation of the effect of ionic radius on the absorbability of the lanthanon ions on cation-exchange columns. They found that the sequence of relative absorbability was the exact reverse of the positions of these elements in the Periodic Table, *i.e.*, lanthanum was the most strongly absorbed and lutetium the least. This is in the same order as the decrease of basicity and increase in the stability of combinations with various chelating agents. It is this greater stability of the heavy lanthanon chelates that may be the basis for their greater skeletal deposition.

Summary. 1. The metabolism of the lanthanons has been studied in the rat on a systematic basis using tracer technics. High-specific activity preparations were administered orally or intramuscularly as citrate complexes. 2. Data are presented for the fifteen lanthanons 1 and 4 days after administration and at intervals up to 8 months for those isotopes with sufficiently long half lives. In general, absorption from the parenteral injection site was relatively complete at four days. Gastrointestinal absorption of the 4 lanthanons administered orally was insignificant. 3. The light lanthanons (lanthanum through samarium). Deposition was primarily in the liver and skeleton, 50 and 25% of the administered dose respectively. Elimination from the liver (presumably by way of the bile duct to the gastrointestinal tract) was quite rapid with a half-time of about 15 days. Two months after injection the skeleton retained about two-thirds of its initial deposition; there was no further elimination from the skeleton during the subsequent 8 months. 4. The transition lanthanons (europium and gadolinium). Deposition was more nearly equal in liver and skeleton, 30 and 40% of the administered dose respectively. Excretion was both fecal and urinary. 5. The heavy lanthanons (terbium through lutetium). Deposition was mainly skeletal, 50 to 65% of the administered dose. Elimination from the skeleton was slow with a half-time of approximately 2.5 years. Excretion of extra-skeletal heavy lanthanon occurred within the first two weeks after injection and was almost entirely urinary.

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Systemic Effects of 2-isovaleryl-1,3-indandione. (22176)

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(Introduced by J. H. Wills.)
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The 2-acyl-1, 3-indandiones possess anticoagulant properties which make the more potent compounds useful as rodenticides(1-4). A typical acyl indandione, 2-isovaleryl-1, 3-indandione (Isoval) was found by Kabat *et al.*(2) to possess no significant effects on blood pressure, respiration, or plasma prothrombin time in cats after slow intravenous administration of 20 mg/kg for 2.5 hours. However, these authors noticed that after oral doses of 100-300 mg/kg given to rats and rabbits, labored respiration, progressive muscular weakness, hyperexcitability, pulmonary congestion, venous engorgement and systolic standstill resulted.

During our study of prolonged plasma coagulation time produced by Isoval in rabbits; we noticed that intravenous injection of comparatively large doses resulted in extremely rapid death (within 2 minutes). Because such an acute effect had not been reported previously it was decided to investigate this action of Isoval and related compounds.

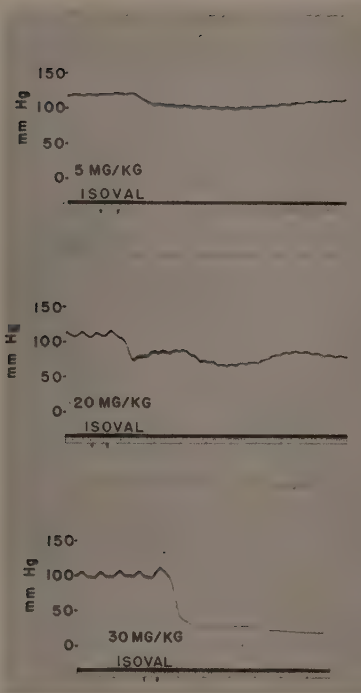
Methods. Cats of mixed breed and sex were used. The animals were anesthetized with pentobarbital and tracheotomized for artificial respiration or for recording respiration by means of Stathan strain gauge attached to pneumotachograph. In some animals, respiratory recordings were made by means of an intrathoracic cannula. The right carotid artery was cannulated and connected to mercury U-tube manometer for blood pressure recording. All injections were made through a polyethylene tube cannula inserted into the right femoral vein. Spinal sections were performed through dorsal incisions at approximately the C-4 level. The sodium salt of Isoval is soluble in water to about 20 mg/cc. When solutions of greater concentration were required, the substance was dissolved in diethylene glycol monoethyl ether. The effect of epinephrine on the Isoval response was determined by giving epinephrine in isotonic saline solutions before, together with, and after injection of the drug.

Results. Fig. 1 a and b show responses obtained at dose levels of 5, 20, 30, 40 and 60 mg/kg Isoval in single cats. The top tracing is respiration measured by means of intrathoracic cannula. As the dose of Isoval was increased, the hypotensive effect became more severe until at higher doses there was no recovery. Concomitant with the drop in blood pressure there was an increase in both rate of respiration and magnitude of intrathoracic pressure variations. Pneumotachographic recordings made on other animals showed that depth of respiration was also increased at higher dose levels.

Giving epinephrine together with and after the drug, had no appreciable effect on ultimate response to the drug. However, when 25 μg/kg of epinephrine was followed by 60 mg/kg of Isoval, a milder response to the drug was seen. In one experiment 25 μg/kg epinephrine was given after blood pressure had dropped to a low level (20 mm Hg) following injection of a mixture of 60 mg/kg of Isoval and 25 μg/kg of epinephrine. No response to this second injection was seen immediately. However, after several minutes, a rapid increase of blood pressure to 100 mm of Hg was seen, followed by a later collapse with ultimate death.

On the assumption that some of the actions of Isoval were mediated centrally, a few experiments were done, in which the injections were given via the carotid artery. However, there seemed to be no change in pattern of blood pressure response from that obtained with intravenous injections.

Another approach, to determine any central effects, involved 3 different types of preparations: Cats with spinal cord sectioned at C-4; cats bilaterally vagotomized; and cats sectioned at C-4 and bilaterally vagotomized.



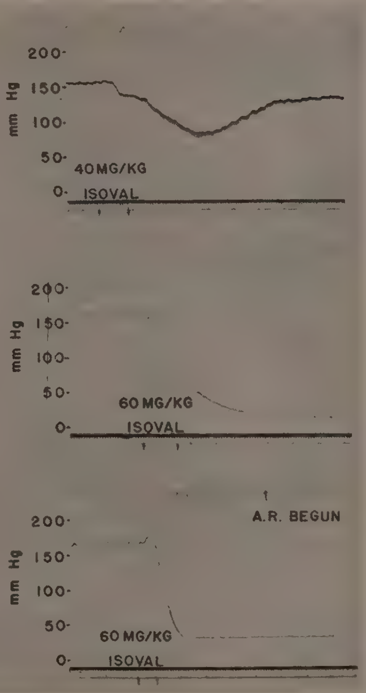


FIG. 1a (left). Blood pressure and intrathoracic respiration recordings at doses of 5, 20, and 30 mg/kg Isoval.

FIG. 1b (right). Blood pressure and intrathoracic respiration recordings at doses of 40 and 60 mg/kg Isoval.

In spinal vagotomized preparations, 2 animals responded to repeated doses of Isoval with only mild decrease in blood pressure and with subsequent recovery to preinjection levels. The first animal received 3 separate doses: 20, 20, 40 mg/kg; the second animals received 40, 40, 20 mg/kg. The first animal ultimately died.

Two similar preparations each received 40 mg/kg Isoval. There was no recovery from blood pressure reduction, which was as rapid as in intact animals.

In neither vagotomized preparations nor spinal sectioned preparations was there any difference in response to Isoval from that seen in intact animals.

Electrocardiographic recordings made of various animals showed no cardiac abnormalities to which the precipitous blood pressure fall could be attributed.

Discussion. When Isoval is administered intravenously to cats in adequate amounts, the principal effects obtained are: (1) rapid decline in blood pressure from which there is no recovery where the dose is large enough and, (2) a concomitant increase in rate and sometimes depth of respiration.

In high spinal-sectioned, vagotomized cats the blood pressure response was in 2 cases identical with that observed in intact animals. In 2 other cats, however, blood pressure decline appeared less drastic for a given dose than that observed in intact animals. Since there was variability even among intact animals in regard to dose required to produce death, it is difficult to determine if any of the spinal-vagotomized animals gave more or less response to a given dose. Vagotomized animals and spinal-sectioned animals with vagi intact did not differ from intact animals in their response to the drug. From these observations it can be concluded tentatively that there is little if any centrally-mediated component in the drug response.

Electrocardiographic recordings showed no marked changes throughout the period of injection of the drug, at which time blood pressure was dropping rapidly. This does not rule out the possibility of a direct action of the drug on the heart. Murtha(5) in some experiments using papillary muscle preparations has been able to show some direct effects of Isoval on the myocardium.

Another possible explanation of the action of this drug is that sudden reduction in blood pressure may be due to rapid vasodilation in the vascular bed. Some slight evidence for this is seen in the fact that when epinephrine was given prior to injection of Isoval, a milder response was observed in some cases. This might imply that the vaso-constrictor action of epinephrine counteracts to some extent the vasodilation of Isoval.

It is interesting to note that Wakim *et al.* (6) used dicumarol and obtained results in dogs similar to ours. They attribute the action of acute doses of dicumarol to generalized vasodilation of the capillaries and small vessels, with a resulting vascular collapse. The fact that dicumarol and Isoval are both anticoagulants suggests that the mechanisms of their acute effects may be identical.

The experiments in which 2-pivalyl-1, 3-indandione (Pival) was used indicated a similar response to that obtained with Isoval. Pival seemed to have a somewhat greater hypotensive effect.

Preliminary experiments with 2-acetyl-1, 3-indandione, 2-phenylacetyl-1, 3-indandione, 2-diphenylacetyl-1, 3-indandione, and 2-benzoyl-1, 3-indandione indicated that these compounds exhibit similar effects. They were not investigated completely because of the difficulty of finding a suitable solvent for intravenous administration.

Conclusions. 1. 2-isovaleryl-1, 3-indandione when given intravenously to cats in doses ranging from 5-60 mg/kg produces a decline in blood pressure which increases in severity with increase in dose. 2. Concomitant with the decline in blood pressure there is an increase in respiratory rate and depth. 3. These effects cannot be attributed to direct action on the heart, nor do they appear to be centrally mediated. 4. It is suggested that rapid vaso-dilation, with consequent drop in blood pressure, may produce a fatal vascular collapse.

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Thiopental and Thyroid Metabolism.* (22177)

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Previous observations(1,2) indicated sodium ethyl (1-methyl-butyl)-thiobarbiturate (thiopental) to reduce the I^{131} uptake by the rat thyroid to 89%. Impairment of thyroid activity was not found related to the anesthetic effect, since anesthetics which did not contain a $N - \dot{C} = S$ grouping did not change the I^{131} uptake(1). Other anesthetic agents containing a thiocarbamate structure have also been shown to have anti-thyroid activity (3). Widespread use of thiopental as an anesthetic agent suggested that its antithyroid properties be further investigated. This study indicates a relationship between metabolism of thiopental by thyroid and ability of thyroid to accumulate I^{131}.

Methods. The rats used in these studies were of the Sprague-Dawley strain, males, 180-200 g. Hypophysectomized rats were all males, average weight 155 g, and were used 26-30 days post operation. The basal ration consisted of commercial grains and water *ad libitum.* Hypophysectomized animals were given a daily supplement of oranges and lettuce. Not less than 8 nor more than 10 animals were used in each experimental group. The I^{131} uptake and assay procedures have been described previously(1,3). Total iodine content of thyroids was determined by modification of the method of McClendon and Foster(4). S^{35} labeled thiopental was obtained from Abbott Laboratories. It was administered in 10 μc doses diluted with non-radioactive thiopental to give animals a 40 mg/kg dose as used in earlier experiments. Analysis of S^{35} in tissues was achieved following oxidation (HNO_3 and $HClO_4$) of tissue sulfur to sulfate which was precipitated with benzidine hydrochloride and assayed in a gas-flow detector. Sulfur content of precipitate was determined by titration of benzidine sulfate with 0.01 N NaOH. Counting was extended to a 2% statistical error. Corrections for decay and self-absorption were applied by conventional methods. Results are expressed as specific activity, defined here as counts per minute (CPM) per μM. In some preliminary experiments, results were expressed as CPM/mg tissue. In the experiment wherein both I^{131} and S^{35} were used in the same animal, the I^{131} was assayed in the thyroids first using a thick-window (3.5 mg/cm^2) G.M. counter which was insensitive to soft beta radiation of S^{35} as determined by previous trial runs with similar amounts of labelled thiopental. Iodine content was determined on pooled single lobes of thyroid while sulfate content was determined on the remaining pooled lobes. In some cases, it was necessary to add known amounts of carrier to effect chemical separation of the sulfate.

Results. Thiopental inhibited thyroid activity immediately following administration (Fig. 1). Response of thyroid in a 4-hour test period was inversely related to the amount of thiopental given to the animal (Table I). Of perhaps even more interest was the observa-

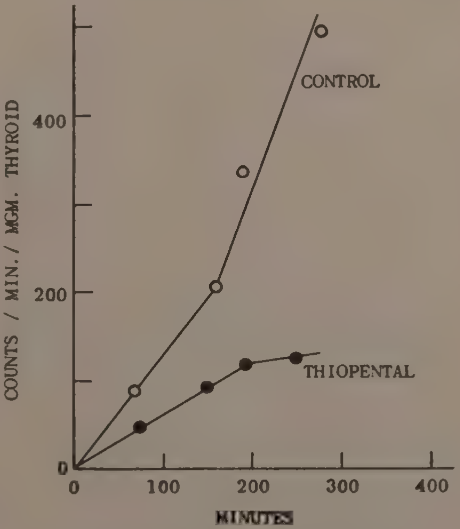

FIG. 1. Effect of thiopental (40 mg/kg) on I^{131} incorporation into rat thyroid; 5 μC I^{131}.

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* These studies were supported in part by a grant from the Office of Naval Research and the English Fund of the Hahnemann Medical College.

TABLE I. Four-Hour Uptake of I^{131} by Rat Thyroid in Relation to Amount of Thiopental Administered. 2 μC I^{131} intraperitoneally.

Dose (mg/kg)	I^{131} CPM/mg	%
0	214 ± 19*	100
5	138 ± 15	64.4
10	112 ± 14	52.3
20	76 ± 9	35.5
40	44 ± 7	20.6
70	26 ± 5	12.2

* Stand. error of the mean.

tion of impairment of thyroid activity following administration of a single 40 mg/kg dose of thiopental (Table II). It required 6 to 7 days for complete recovery. To obtain information on the nature of the inhibition, I^{131} uptakes were performed on hypophysectomized animals. The results shown in Table III indicate that thiopental reduces the uptake of I^{131} in the absence of the pituitary and suggest that the principal mode of action is via interference with iodine uptake, probably by reduction of I_2 to I^-(5). In another experiment, normal animals were given I^{131} and samples were taken for thyroid assay at 24 hours. Remaining groups were given thiopental (40 mg/kg) and thyroids assayed(1-3) 24 hours later. Compared to controls the treated animals lost I^{131} rapidly (Fig. 2). A final experiment was performed wherein S^{35} labelled thiopental was administered along with carrier-free I^{131} to intact and hypophysectomized rats. The results indicated that recovery of the thyroid was related to disappearance of S^{35} from thiopental in the thyroid (Fig. 3). The S^{35} content of pituitary and adrenal seemed to bear no relation to thyroid iodine metabolism. Specific activities of the normal rat adrenal S^{35} at 2, 24, 48, 120, and 144 hours post thiopental were 93.5, 7.9, 2.4, 2.0, and 1.8; of the pituitary 4.1, 2.6, 1.9, 2.2. The levels of S^{35} from thiopental in the endocrines of the hypophysectomized rats were lower than controls and a constant level of S^{35} in the thyroid of hypophysectomized rats appeared to be related to a constant I^{131} uptake (Fig. 3). The specific activities of the hypophysectomized rat adrenal S^{35} were 4.4, 2.0, and 1.8 at 24, 48, and 120 hours.

TABLE II. Recovery of I^{131} Trapping Ability of Thyroid following a Single Dose of Thiopental (40 mg/kg).

Hr	I^{131} uptake (% controls)
4	18
12	33
24	47
48	56
72	62
96	64
168	96
192	100

TABLE III. Effect of Thiopental on I^{131} Uptake by Thyroids of Hypophysectomized and Normal Rats.

Group	CPM/mg	%
Hypox	28.8 ± 5.7*	100
Hypox + thiopental	6.8 ± 0.7	23.6
Normal	258.0 ± 25.4	100
Normal + thiopental	30.1 ± 4.7	11.6

* Stand. error of the mean.

Discussion. The data presented here suggest that thiopental possesses properties of an antithyroid substance predictable by its thiocarbamate structure. That it also possesses anesthetic properties is indeed most interesting. Prolonged inhibition of thyroid activity demonstrated by a single dose is of interest in view of the observations of others(6,7) that thiopental is rapidly removed from the blood stream and stored in body fat from which it is slowly released. Further, studies on metabolism of thiopental(7,8) indicate it to be diversely attacked by the organism, thiourea being one of the many metabolic products. It may be that thiourea or substituted thioureas formed by metabolism of thiopental may be ultimately responsible for the prolonged in-

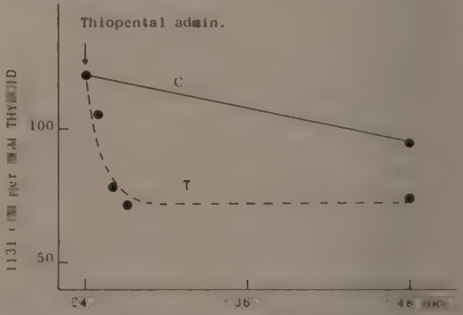

FIG. 2. Reduction of I^{131} content by administration of thiopental (40 mg/kg) to rats given 1 μC I^{131} 24 hr previously.

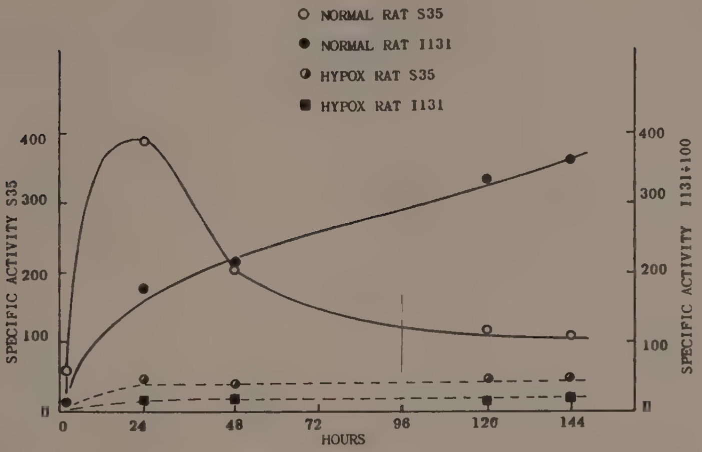

FIG. 3. Thyroid I^{131} in relation to S^{35} from thiopental in normal and hypophysectomized rats.

hibition. The relatively long period wherein activity from the S^{35} thiopental was found in the thyroid (Fig. 3) supports this hypothesis. The S^{35} activities of the pituitary and adrenal show no obvious relation to inhibition of the thyroid suggesting that other endocrines are not involved. The S^{35} and I^{131} specific activities of thyroids of hypophysectomized rats were both low and did not change during the experiment. Since thyroid in these animals was not functioning properly due to absence of the thyrotrophic hormone, these results were not unexpected.

In vitro studies by investigators indicated that aqueous thiopental solutions reduced I_2 to I^-. The reduction was not stoichiometric, more than molar equivalents of I_2 were reduced by thiopental. These observations suggest that the reaction is quite complex. This phenomenon is characteristic of many antithyroid agents(9). It would appear that thiopental could deprive the thyroid of I_2 by reducing it to I^- in the organism. This view is supported further by the reduced uptake of I^{131} in the hypophysectomized rat treated with thiopental. The ratio of inorganic I^{131} to diiodotyrosine I^{131} to thyroxine I^{131} was 54; 33; 13, and 57; 28; 15 in treated and nontreated animals respectively (4-hour uptake). These nearly identical ratios of activities indicated that thiopental did not interfere with thyroxine synthesis but functioned by depriving the thyroid of iodine. Plasma PBI^{131} in thiopental-treated rats was also reduced to 49% of control values, another indication that the primary action is a reduction of available I_2 for synthetic purposes.

Summary. 1. Thiopental influences thyroid metabolism primarily by depriving the thyroid of available I_2. 2. The prolonged inhibition of thyroid activity by thiopental is probably related to accumulation of its metabolic products in the thyroid.

We wish to acknowledge the technical assistance of Evelyn Repplinger and Robert L. Shermann.

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Some Properties of Human Serum Lipoproteins.* (22178)

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Our concern with the lipides of serum has been principally with respect to their characterization as lipoproteins. In this regard we have been able to demonstrate, by a variety of technics, that carotene, acetal phosphatidyl choline and probably sphingomyelin are *characteristic* of human β_1-lipoprotein(1,2); carotene had already been shown by others (3) to be associated with β_1-lipoprotein. The presence of acetal lipides in serum is not a new observation(4); only recently, however, have studies directed toward demonstrating the protein association of serum acetal phosphatides been reported(1,5). We have been unable to demonstrate a lipide component characteristic of α_1-lipoprotein but do find vit. A to be regularly associated with albumin.

Methods. Preparative Ultracentrifugation. 200 to 400 ml of blood were obtained by veni-puncture from normal, postabsorptive, human subjects and let clot undisturbed at 20°C for an hour. The serum was separated by centrifugation in an International refrigerated centrifuge (15°) and promptly submitted to ultracentrifugation for 8-10 hours at 4-12° using a Spinco E5 rotor at 52640 rpm (ca 200000 g). Appearance of the tubes after centrifugation is diagrammed in Fig. 1. Fractions 1-5 were quantitatively collected by means of a specially constructed aspirating device and examined by one or more of the following procedures: 1) Refractionation in the ultracentrifuge after adjusting density to (a) 1.063 with NaCl or to (b) 1.21 with NaCl and KBr or with D_2O and NaCl. Four to 6 hours at 200000 g with the E5 rotor sufficed to float the desired fraction (β_1-lipoprotein at 1.063 and both α_1 and β_1-lipoproteins at 1.21) into the top ml which was collected by aspiration. 2) Analytical schlieren analyses of all samples were performed after adjusting the density to 1.063 or to 1.21. For these analyses the Spinco An-A rotor operating at 52640 rpm was employed, utilizing both visual inspection of the viewing screen and photographs in the analysis. 3) Chemical analysis on (a) chloroform:methanol (4/1:v/v) extracts and (b) benzene extracts of frozen-dried material. Phosphorus and choline were estimated as described earlier(6); cholesterol(7); total lipide esters (8); carotene from its absorption spectrum (9); and vit. A using both the Carr-Price reaction(9) and the technic of Bessey, *et al.* (10). As here measured choline was an estimate of the lecithin (phosphatidyl choline) and of acetal phosphatidyl choline; sphingomyelin may be estimated by difference from

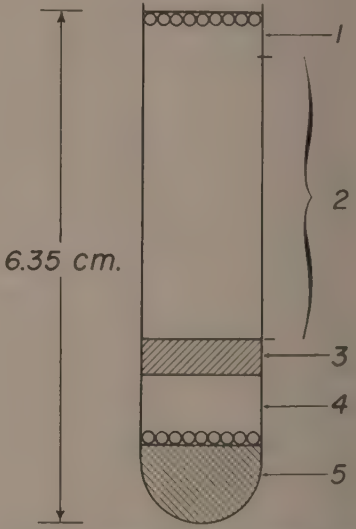

FIG. 1. Serum following 8 hr ultracentrifugation; (1) water-clear to milky; (2) water-clear; (3) bright orange-yellow, turbid; (4) yellow, clear; (5) yellow-brown, clear and includes thin flocculent layer dividing 4 from 5. Typical volume recoveries are 0.6, 3.6, 0.5, 0.7, and 1.0 ml respectively.

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TABLE I. Typical Data from Chloroform : Methanol and Benzene (in Parenthesis) Extracts of Frozen-Dried Serum Fractions Obtained by Ultracentrifugation as Described in the Text.

		Phosphorus		KOH hydrolyzable choline	Cholesterol		Total carboxylic esters			
		10^{-4} mM/ml fraction								
Fraction	Color of extract*	Total	KOH hydrolyzable		Total	Non-esterified		Carotene	Vit. A	Acetal lipide
1	W (W)	14.4 (6.3)	12.3	0	10.0 (10.0)	5.1 (5.0)	198 (163)	0 (0)	0 (0)	0 (0)
2	W (W)	4.4 (0)	4.3	0	3.1 (3.0)	2.0 (2.0)	63 (38)	0 (0)	0 (0)	0 (0)
3	Y (Y)	150.0 (4.8)	113.2	85.0	253.1 (171.3)	56.2 (55.7)	735 (200)	4+ (4+)	1+ (0)	4+ (±)
4	Y (W)	64.0 (0)	55.1	38.8	54.4 (1.3)	9.0 (1.1)	270 (0)	± (0)	2+ (0)	+ (±)
5	Y (W)	38.5 (0)	33.8	19.4	28.3 (1.0)	3.1 (0.5)	145 (0)	0 (0)	4+ (0)	± (±)

* W = White; Y = Yellow.

the total and KOH phosphorus values(6). The desirability of using frozen-dried preparations in a study of this kind has been elaborated elsewhere(11). 4) Paper chromatographic analysis of both chloroform: methanol and benzene extracts(12). 5) Paper electrophoresis (ionophoresis) both a Kunkel-type apparatus and a Spinco Model R were used at pH 8.6 (0.05 *M* sodium barbital) usually operating at 250 volts, 2 m.a. for 20 hours. Whole serum was submitted to the techniques described above to aid in interpretation of the fraction data.

Results. With respect to their lipoprotein content, fractions 1 and 2 were normally quantitatively insignificant, together accounting for only 7% of the serum cholesterol and 14% of the phosphatide although occupying 66% of the volume. Fraction 1 consisted of chylomicrons which were principally triglyceride and cholesterol(2), all of which was readily extractable from frozen-dried samples by benzene, while only 50% of their phosphatide was so extracted (Table I). Fraction 2 was characterized by its low lipide content; it should be recognized that this fraction is adjacent, above and below, to fractions of high lipide content (Fig. 1).

Fraction 3 usually contained all of the β_1-lipoprotein and perhaps 50% of the α_1-lipoprotein as judged by the schlieren tracings (Fig. 2). The β_1-lipoprotein was easily separable from α_1-lipoprotein by ultracentrifugation at density 1.21. This separation was accomplished in the An-A rotor; centrifugation continued until, by inspection of the viewing screen, the yellow β_1-lipoprotein peak disappeared to the left; the α_1-peak was then nearly halfway across the field. Removal of the analytical cell from the rotor permitted aspiration of the subnatant solution which

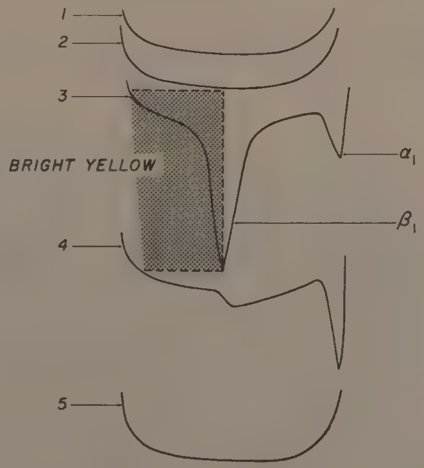

FIG. 2. Flotation schlieren patterns (22 min. pictures) from fractions 1-5 after adjusting density to 1.21 with $D_2O \cdot NaCl$. Occasionally an α_1-peak was observed in 5. When adjusted to density 1.063 the β_1-peak had an S_f of 6. The yellow color of the β_1-peak in curve 3 was readily observed on the viewing screen during the run.

TABLE II. Representative Cholesterol-Phosphatide, Free-Total Cholesterol Mole-Ratios of Various Human Blood Lipoprotein Fractions: (a) This Paper, (b) Others.

Source	Cholesterol / Phosphatide	Free cholesterol / Total cholesterol
a. Fraction 1	.69	.51
2	.68	.51
3	1.9	.27
4	1.0	.20
5	.75	.14
$D_2O \cdot NaCl$ β_1	2.8	.28
α_1*	1.6	.25
Diabetic β_1†	1.8	.49
b. Cohn fractionation(3)		
β_1	2.1	.27
α_1	1.4	.27
Starch ionophoresis (13)		
β_1	2.1	.27
α_1	.58	.22
Ultracentrifugation(3)		
$\beta_1(S_f4)$	2.4	.25

* Shown not to be free of β_1.

† Obtained by preparative ultracentrifugation of serum from patient in diabetic acidosis. This material collected at the top of the tube as an orange-yellow waxy layer similar to Hunter's(2) cream-layer β_1.

contained the α_1-lipoprotein and some β_1. The bulk of the β_1-lipoprotein remained on the upper face of the cell as an orange wax, easily lifted off; these materials were then examined by ionophoresis, paper chromatography, etc. (Table II). As in the case of ionophoresis of fraction 3, these β_1 samples migrated more rapidly than the β_1-lipoprotein in whole serum (Fig. 3), probably due to the absence of *plain* β_1-globulin. In all cases β_1-lipoprotein was characterized by intense osmiophilia, evidently due to the carotene, and by a strong reaction for acetal lipide. By means of various spot-tests and paper chromatography(12) the major acetal lipide component of β_1-lipoprotein was identified as acetal phosphatidyl choline(2,12). The α_1-lipoprotein was identified through its paper electrophoretic mobility on staining with bromophenol blue; it was not osmiophilic and did not stain for acetal lipide. Albumin and non-lipide globulins were absent from the $D_2O \cdot NaCl$ preparations.

Fraction 4 usually contained the rest of the α_1-lipoprotein as demonstrated by ionophoresis and by ultracentrifugation at density 1.21 in $D_2O \cdot NaCl$. Similarly fraction 5 was shown to contain most of the vitamin A (osmiophilia in the albumin region, *cf.* Fig. 3) and occasionally some α_1-lipoprotein. In the $D_2O \cdot NaCl$ refractionation the vitamin A was found to be below the top milliliter.

Ionophoretically, fractions 1 and 2 were negative except for traces of albumin. Fraction 3 showed a trace of γ-globulin, all the β_1-lipoprotein, some α_1-globulin (lipoprotein?) and a moderate amount of albumin. Fraction 4 showed a moderate amount of γ-globulin, no

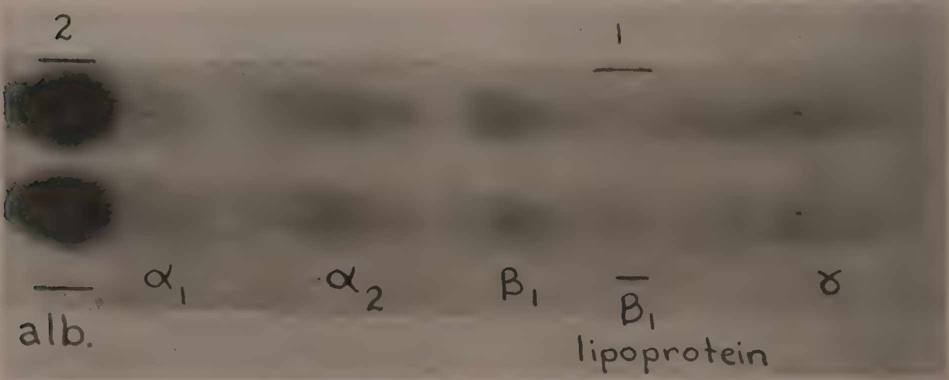

FIG. 3. Ionophoresis of 10 lambda aliquots of 2 normal human sera. The paper was dried promptly at room temperature by means of a fan and exposed to OsO_4 vapors for 15 min.(12) resulting in a browning in the zones delineated by the pencil lines 1 and 2. The paper was then stained for protein using bromophenol blue. On duplicate specimens it was demonstrated that usually only the β_1 osmophilic region was positive for acetal lipide.

β_1-lipoprotein, considerable β_1, α_2 and α_1 globulins and albumin. Fraction 5 was similar to 4 except for markedly diminished α_1 and a marked increase in the other globulins and albumin.

Discussion. The behavior of frozen-dried β_1-lipoprotein in chloroform: methanol and in benzene as here described (Table I) renders less useful the notion that *all* protein-bound lipides can be estimated by the solvent partition method(6,12). In this case carotene and cholesterol, for example, seem to be loosely bound, *i.e.*, extractable by benzene. On the other hand it is apparent that the other blood lipoproteins (excepting chylomicrons) are more resistant to benzene extraction.

Summary. Normal, postabsorptive, human serum was fractionated by means of the ultracentrifuge and the lipoproteins studied with the aid of paper chromatography, ionophoresis and by chemical procedures. The characteristic features of β_1-lipoprotein and methods for its isolation are described.

I am grateful to Beatrice Gaupp, Marian McCutcheon and Ediberto Blasini for skilled technical assistance.

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Development of Arthritis, Periarthritis and Periostitis in Rats Given Adjuvants.* (22179)

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In this investigation skeletal muscle was incorporated into a Freund-type water-in-oil emulsion and the mixture administered by various routes to rats. Within a short time, ranging from 14 to 45 days, a high percentage of the animals which received intracutaneous inoculations began to develop tender swellings of the ankles, the paws, the tail and the digits. Single or multiple joints were involved. The experiment was repeated on two further occasions. In the third trial a different strain of rats was used. The original results were readily reproducible.

Materials and methods. Originally both male and female rats of the Wistar strain were used. They ranged in weight from 230 to 280 g. The final series contained female rats of the Long-Evans strain, weighing 180 to 220 g. All animals were fed a standard pellet diet of Purina chow and water *ad libitum*. They were weighed frequently and inspected almost daily throughout the investigation period. The inoculum was prepared as follows: a healthy rat of the same species to be subsequently used in the study was killed

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TABLE I. Incidence of Clinical Alteration of Extremities and Tail in Relation to the Site of Inoculation—Series 55A1.

Site of inoculation	No. of rats (Wistar)	Severity of change				% animals affected
		None	Slight	Moderate	Severe	
Intracutaneous (posterior cervical)	17	4	6	2	5	77
Subcutaneous	8	6	1	1	0	25
Intraperitoneal*	4	0	0	0	0	0
Controls (no inoculations)	8	8	0	0	0	0

* Produced fibroplastic peritonitis but no peripheral alteration.

Mean time of alteration after initial injection—23 days (15-32 days). Avg No. of injections before alterations—2.9 (1-5).

by a sharp blow. Skeletal muscles from the thigh and the iliopsoas groups were freed from as much surrounding fat and connective tissue as possible and weighed in a sterile beaker. Ten grams of this muscle was homogenized in a Waring micro-blendor with 30 ml of sterile isotonic saline solution using intermittent action of the rotor blades to avoid overheating of the mixture. The resultant homogenate was then compressed through fine-mesh sterile gauze and to the filtrate was added 32 ml of refined thin mineral oil, 8 ml of the emulsifier Falba,[*] and 100 mg of heat-killed *Myco. phlei*. The mixture was emulsified by the syringe method in a sealed vial and stored at 4°C. It was warmed to 50°C for a few minutes each time inoculations were given. In the final series aqueous Merthiolate, to a final dilution of 1:10000, was added without significant effect on the final result. In one portion of the final experiment muscle tissue was excluded from the inoculum and was replaced by an approximately equal amount of sterile isotonic saline solution. In another portion of this experiment only saline and muscle were combined so that the final concentration of muscle homogenate was approximately the same as in the mixture with adjuvants added. All animals were given 0.1 ml of the emulsion at each inoculation. Injections were given in a series of 3 at 2-day intervals, then a 14-day rest period was followed by a second and in some animals a third and fourth series of injections. In the first experiment inoculations were given intracutaneously into the skin of the posterior cervical region after first carefully clipping the hair. Subcutaneous injections were also given into this region. A small group of animals were given intraperitoneal injections. In the second and third experiments all inoculations were given intracutaneously. A 21 gauge needle and tuberculin syringe were used for all injections.

Results. A total of 97 animals were used in the study which was divided into 3 experiments. The results of each will be presented separately but the discussion of the clinical and pathological changes will be combined since they were essentially similar in all groups. A detailed autopsy was performed on every animal that has been sacrificed. Some animals showing clinical change have been allowed to recover and are under continued observation.

Controls. Unrelated Deaths. Uninjected control animals from the same litter groups as the treated animals have been followed in each experiment. Clinically and at autopsy these have consistently failed to demonstrate any lesions of the joints. Two animals in the treated and one in the control group died from acute pneumonitis.

First Exp. Wistar rats were given inoculations by various routes. The results are shown in Table I and demonstrate the superiority of the intracutaneous mode of injection. The incidence and degree of response was similar in both sexes. *Second Exp.* The initial experiment was repeated, again with Wistar rats and a freshly prepared inoculum. All injections were given intracutaneously and the initial results were essentially reproducible as shown in Table II. *Third Exp.* Long-Evans female rats were used and the content of the inoculum was varied to determine the

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[†] Obtained from the McNerney Chemical Corp., Los Angeles, Calif.

TABLE II. Incidence of Clinical Alteration of Extremities and Tail—Intracutaneous Inoculations.

Series	No. of rats (Wistar)	Severity of change				% animals affected
		None	Slight	Moderate	Severe	
55A 13-15	16	6	4	4	2	62
Controls	8	8	0	0	0	0

Mean time of alteration after initial injection—28 days (20-45 days). Avg No. of injections before alteration—4.1 (3-6).

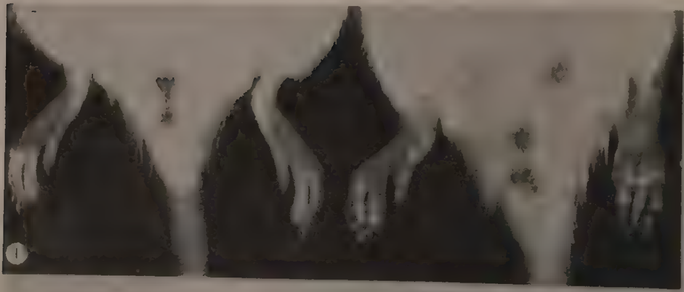
relative value of various components in inciting the pathologic changes as noted. The results show that although muscle tissue does not appear to be necessary for the development of these changes it may have a potentiating effect. Acid fast bacilli and probably the mineral oil are essential. The incidence of clinical alteration in this experiment is shown in Table III.

Time of Onset and Clinical Appearance of Limb and Tail Alterations. The earliest alterations occurred on the fourteenth day after the original inoculation. Initial alterations in other animals were delayed up to 45 days. The average time of onset was 22 days. The first changes usually were seen in the ankles, hind paws or proximal or middle phalangeal joints of the feet. In general the earlier the onset the more severe the clinical involvement but this was not always the case. In a small series, not included above, only one intracutaneous inoculation was given and 3 out of 8 animals have shown joint alterations. The ankles and tarsal regions were most frequently affected severely; then the forepaws, digits and tail. When markedly affected, serum exuded from the swollen red paws which were obviously painful and tender to touch. Frequently one or several digits would be completely spared from swelling although adjacent ones were 2 or 3 times their normal size (Fig. 1). The tail was more frequently involved in the Wistar strain and showed swelling, nodulation and desquamation, more marked in the proximal segments. Many severely affected animals lost weight. Lesser degrees of swelling affected many other animals and again the ankles were most frequently involved. Fusiform red swelling of one, several or all of the proximal interphalangeal joints was not uncommon (Fig. 2) especially in the Wistar strain. Diffuse digital swelling was more commonly seen in the Long-Evans strain. In animals that were observed for several weeks or months there was a gradual return of lost weight as evidence of acute inflammation subsided. Functional recovery did not continue beyond 3 months at which time there were still many residuals in the form of chronic firm enlargements especially about the ankle joints (Fig. 4), the metatarso- and metacarpophalangeal joints which showed typical flexion deformities (Fig. 3) resembling a "flipper hand," and large rigid tails. Firm ankyloses often partially or almost completely restricted motion especially at the ankles and in the tail joints. Histological study has not as yet been made on these chronic alterations. *Roentgenologic Observations.* After one month, early bone

TABLE III. Incidence of Clinical Alteration of Extremities and Tail with Varying Composition of Inoculation Material—Series 55A16-21.

Inoculum content	No. of rats (Long-Evans)	Severity of change				% animals affected
		None	Slight	Moderate	Severe	
Adjuvant + muscle + merthiolate (1:10,000)	8	3	2	1	2	62
Adjuvant alone*	8	5	2	0	1	37
Adjuvant (no acid fast bacilli) + muscle†	8	8	0	0	0	0
Saline + muscle	6	6	0	0	0	0
Controls (no inoculations)	6	6	0	0	0	0

* Small granulomas at injection site. † No granulomas at injection site.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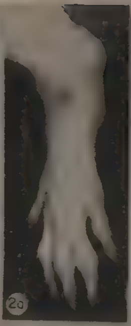
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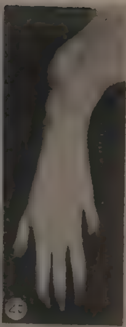
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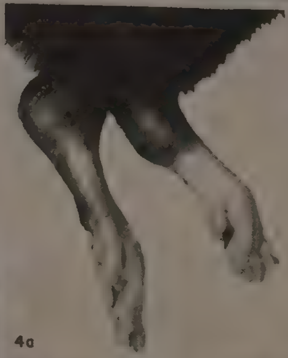
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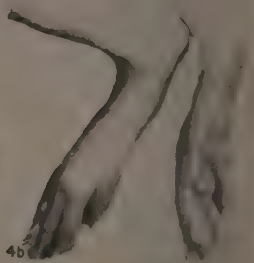
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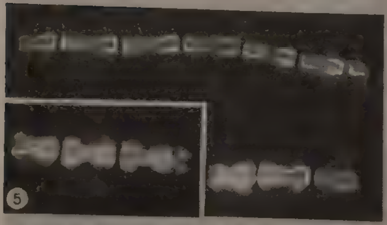
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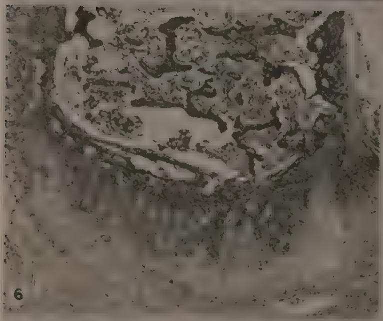
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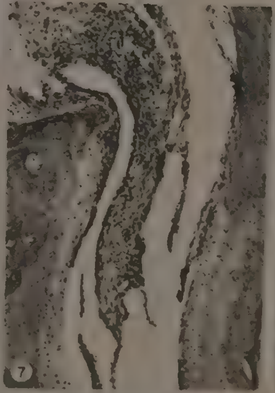
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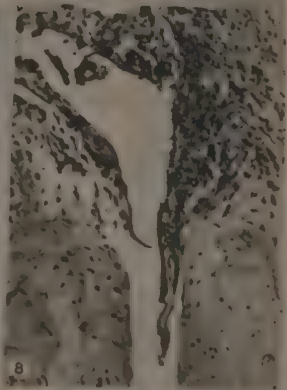
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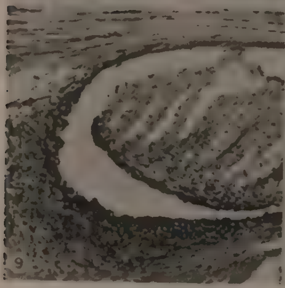
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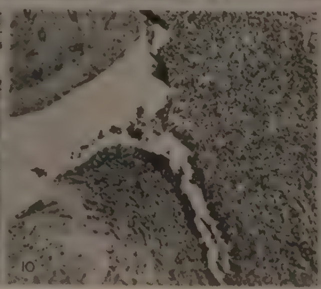
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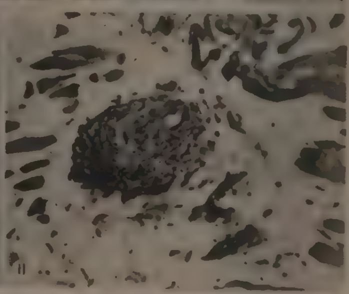
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FIG. 1. Diffuse swelling of both tarsal regions and digits compared with control animal. Note absence of involvement of middle toes.

FIG. 2. (a) Fusiform swelling of proximal interphalangeal joints. (b) Control.

FIG. 3. Flexion deformities at metacarpo- and metatarsophalangeal joints. Duration of disease, 4 mo. Retouched photograph.

FIG. 4. (a) Chronic firm swelling of ankles with marked restriction of ankle motion. (b) Control.

FIG. 5. X-rays of tail vertebrae. The upper picture is a control. Lower X-ray demonstrates increased periosteal density, exostoses and narrowing of intervertebral disc spaces.

FIG. 6. Section through os calcis showing active periosteal osteogenesis and inflammatory reaction on the right with invasion by granulation tissue through the bony cortex and into the marrow cavities in this area. × 45 (before reduction).

FIG. 7. An ankle joint showing a broad, edematous, inflamed synovial villus projecting into the joint space. Note extension of granulation tissue over the surfaces of the articular cartilage. × 180 (before reduction).

FIG. 8. Fibrinoid necrosis of synovial margin of a tarsal joint. × 220 (before reduction).

FIG. 9. Peritendonitis and tendonitis. × 130 (before reduction).

FIG. 10. Intense inflammatory reaction, granulation tissue growth and synovial reduplication at the margin of a phalangeal joint. Note neutrophiles in joint space. × 180 (before reduction).

FIG. 11. Perivascular lymphocytic collection about a small intramuscular artery in one of the small muscles of the foot. × 220 (before reduction).

and joint changes were visualized. These were progressive during the "healing" phase and were maximal at 3 months. They were most marked about the os calcis and the ankle joint but were often intense on the lateral wings of the caudal vertebrae (Fig. 5). The change consisted essentially of a periostitis followed by the appearance of nodular exostoses along the entire surface or an isolated segment of bone. The joint spaces of the ankle were narrowed or completely obliterated and the intervertebral spaces of the tail were narrowed. Oblique views of formalin-fixed spine showed some bony reaction with partial or complete obliteration of the apophyseal joint spaces in the lower dorsal and lumbar spine. No other joints were altered radiographically.

Histopathologic Observations. Animals were sacrificed at various stages during the development and subsidence of the clinically observed bone and joint lesions. The process was essentially one of intense stimulation of the connective tissue elements with focal and diffuse inflammatory infiltrations. This response was limited to the regions about the affected bones, joints and tendons and rarely extended into the subcutaneous tissue. The overlying dermis was normal. Edema, fibroblastic proliferation and sparse mononuclear or polymorphonuclear infiltrations were noted in the earliest stages. After 10 or more days there was exuberant proliferation of granulation tissue about some bones, joints, tendons, synovial sheaths and synovia of the joints. This tissue was responsible for the focal swellings and hyperemia seen clinically. It was infiltrated to a variable degree, often intensely, with neutrophiles, plasma cells, lymphocytes and mononuclear histiocytes. Eosinophiles were rarely seen. The next most striking observation was the profound osteoblastic activity which arose mainly from the periosteum of cortical bone and rapidly formed broad spicules of new bone (Fig. 6). This bone was frequently surrounded at its outer border by a zone of immature cartilage which appeared to arise *in situ* in the adjacent hyperplastic connective tissue. Granulation tissue frequently invaded the cortical bone and spread out into the marrow cavity (Fig. 6). The synovia about tendon sheaths and joint spaces was hyperplastic and portions of tendon were often invaded (Fig. 9) and adherent to the side of the sheath. The synovial villi were enlarged, edematous and inflamed and frequently projected deeply into the joint spaces (Fig. 7). Occasionally fibrillar material surrounded the joint margin and resembled fibrinoid necrosis (Fig. 8). Small numbers of neutrophiles were present in some joint spaces (Fig. 10) along with an apparent slight increase in joint fluid. There was no primary involvement of the articular cartilages but granulation tissue from the margins of the synovial membrane was frequently observed growing over the surfaces of the articular cartilages (Fig. 7). The above changes were seen in the ankle joints or the joints of the paws or fingers when clinical involvement was apparent. Lesser degrees of connective tissue reaction were occasionally noted about bones and joints not grossly affected.

Sections of the tail showed an essentially similar process plus early invasion of the margins of the intervertebral discs and the subchondral spaces by inflamed granulation tissue. Other findings of interest were focal lymphocytic and mononuclear cellular infiltrates in skeletal muscles near involved bones and joints. This was a true focal polymyositis with destruction of one or several striated muscle fibers. An arteritis or vasculitis was infrequently noted in the regions of bone and joint involvement (Fig. 11). It consisted of lymphocytic and mononuclear cellular infiltrates about a portion or all of a small intramuscular artery. There was no necrosis of the vessel walls. Focal areas of edema and necrosis, without surrounding palisading, were occasionally seen in the hyperplastic connective tissue. Occasional focal subcutaneous lymphocytic collections were noted.

Similar histologic changes were not noted in muscle and other tissues remote from the areas of clinical involvement. All other organs were histologically negative except for an occasional small granulomatous focus in the liver and varying degrees of a granulomatous pneumonitis in some animals. Similar pulmonary lesions have been frequently noted in guinea pigs following repeated injections of a Freund-type adjuvant(1). In summary the pathological changes consisted of: connective tissue proliferation and granulation tissue formation; acute and chronic inflammatory infiltrates; periostitis with periosteal and enchondral new bone formation; arthritis and periarthritis; synovitis; tendonitis and peritendonitis; focal myositis (near affected bones and joints only); perivasculitis, non-necrotising (rare).

Discussion. There have been many previous attempts to produce an arthritis in animals. Some of these have been successful by such varied methods as injecting large amounts of desoxycorticosterone acetate into rats previously nephrectomized and adrenalectomized(2), inoculating rabbits repeatedly with large amounts of horse serum(3) or killed beta hemolytic streptococci plus chondroitin sulfate(4), and by injection of viable pleuropneumonia-like micro-organisms into mice and rats(5).

The pathogenesis of the bone, joint and connective tissue alterations reported herein must at present remain a matter of speculation. There is suggestive evidence that hypersensitivity is an important factor in these reactions when it is considered that a known allergenic emulsion was used as the inciting agent. Studies are underway to determine if activation of pleuropneumonia-like microorganisms are responsible for these reactions. Periosteal osteogenesis has not been recorded as a feature of this infection, however it is a prominent factor in experimental lathyrism (6).

Pathologically the basic lesion appears to be an intensive focalized mesenchymal tissue response. There are several gross and microscopic similarities between these lesions and those observed in human rheumatoid arthritis but since it is likely that such tissues can only react in a limited number of ways to many stimuli the identification of such changes with any human counterpart is unwarranted.

Summary and Conclusions. A reproducible generalized or focal arthritis, synovitis, periostitis and tendonitis has been induced in rats by intracutaneous injections of a Freund-type adjuvant which was most potent when macerated striated muscle tissue was added to the inoculum. The pathogenetic mechanisms underlying the production of these changes is at present unclear.

The author is indebted to Dr. A. S. Rose, University of California Medical Center, for continuing encouragement and support. Gratitude is also extended for technical assistance of Ruth Lackey and to E. Taylor Peterson of Department of Bacteriology, Wadsworth Hospital, Los Angeles Veterans Administration for the *Myco. phlei* used.

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Plasma Thromboplastin Component: Influence of Coumarin Compounds and Vitamin K on Its Activity in Serum. (22180)

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A new blood clotting factor, termed plasma thromboplastin component (PTC), was recently described by Aggeler, *et al.*(1) and subsequently called Christmas factor by Biggs, *et al.*(2). Little is known about the formation of PTC or about its disappearance from the blood stream. Early workers demonstrated that the level of prothrombin in the blood stream is influenced by vit. K and the coumarin compounds. Since these compounds have been shown to influence "stable factor" as well(3,4), it is of interest to determine whether PTC is similarly affected. The present study demonstrates that patients deficient in vit. K develop a depression of PTC, and confirms the observation of Sise, *et al.*(5) that following the administration of the coumarin compounds there is also a depression of PTC. In both groups we find that PTC activity is restored, in part at least, by the administration of vit. K analogues.

Methods. One-Stage Prothrombin Time was determined by the Link-Shapiro(6,7) modification of Quick's method(8), using commercial rabbit brain thromboplastin* (normal: 14 seconds). *One-Stage Prothrombin Activity* ("per cent" of normal). Normal oxalated plasma was serially diluted with saline (90%, 80%, etc.), and the one-stage prothrombin times of various dilutions determined. Observed times were then plotted against dilution, to provide a standardization curve for comparison of unknown plasmas with normal ones. *Assay of Hemophilioid Factors; Principles Involved*: Thromboplastin is essential for normal conversion of prothrombin into thrombin. Thromboplastin obtained from rabbit brain will react with normal recalcified plasma to form a clot in 14 seconds. This is the basis of the one-stage prothrombin time test. Washed human platelets can form an equally potent thromboplastin, but unlike rabbit brain, must first be incubated with the hemophilioid factors and calcium(9). Hemophilioid factors include: plasma thromboplastin component (PTC), plasma thromboplastin antecedent (PTA) (10) and anti-hemophilic factor (AHF). The ability of these factors, in combination, to transform platelets into a potent thromboplastin, the equivalent of that obtained from rabbit brain, is the basis for the thromboplastin generation test, Biggs, *et al.*(9,11). In the present experiments the thromboplastin generation test is modified so that it becomes a quantitative rather than merely a qualitative assay. Thus the relative levels of any one of the hemophilioid factors can be determined. In the quantitative procedure a reaction mixture containing platelets and calcium is supplied with two of the hemophilioid factors, each in high titer. The third factor—the one to be assayed—is added as an unknown or test preparation. A deficiency of this third factor will be demonstrated by faulty "activation" of thromboplastin. Of the three possible assay procedures, only one was used in the present study—the assay for PTC. *Details of the PTC Assay*: A standard platelet suspension is obtained by the differential centrifugation of citrated normal human plasma(9). Adsorbed plasma is prepared by adding 0.2 cc aluminum hydroxide suspension(12) to 2.0 cc of citrated human plasma, agitating for 5 minutes at 37°C, and centrifuging at 2500 rpm for 15 minutes. The supernatant plasma is then removed and diluted 1:5 with 0.85% saline. Serum is prepared by incubating clotted human blood at 37°C for 90 minutes. To insure uniformity, reagents are then frozen and stored at –40°C until used. Sera provide the PTC and PTA needed to activate the platelet suspension; adsorbed plasma provides the AHF. A test serum under investigation, or a normal control serum, is the source of PTC. The assay is based upon variations in PTC activity supplied by such sera. These sera also contain PTA, but unknown sera cannot be used as a reliable source of this factor, since PTA levels in them may vary. We therefore make it a practice to add a stabilizing surplus of PTA, in the form of serum from a patient rich in PTA but congenitally deficient in PTC. Finally, a stabilizing amount of AHF is likewise supplied by adding normal plasma, adsorbed with $Al(OH)_3$; by virtue of the adsorption, this plasma is almost entirely free of PTC and prothrombin.

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*Prepared by Warner-Chilcott Laboratories.

The reagents are then mixed. An appropriate amount of saline is included in Sol. A to increase sensitivity of the assay.

Sol. A: .10 cc test serum (unknown or normal control serum); 8.20 cc .85% saline; .46 cc serum from patient congenitally deficient in PTC but rich in PTA; 1.24 cc 15% calcium-free acacia in .85% saline to maintain colloidality.†

Sol. B: .20 cc normal $Al(OH)_3$ adsorbed plasma, diluted 1:5 with .85% saline; .20 cc human platelet suspension; .20 cc .02 M $CaCl_2$. Incubate 0.20 cc of Solution A with .60 cc of Solution B in a test tube at 37°C. In this mixture, the activity of thromboplastin reaches a peak after 3-6 minutes of incubation; it then diminishes. Since the amount of PTC in the incubation mixture limits the level reached by this peak, the peak level was chosen as an appropriate measure of PTC. To detect the topmost level with certainty, determinations of thromboplastic activity are made periodically in the region of the peak. At one minute intervals, 0.10 cc aliquots of the incubating thromboplastin are added, simultaneously with 0.10 cc of 0.02 M $CaCl_2$, to 0.10 cc of normal citrated plasma and the clotting times recorded. The citrated normal plasma provides labile and stable factors, prothrombin and fibrinogen. The shortest clotting time of the mixture—recorded at the moment of maximal activation—provides a measure of peak thromboplastic activity. It is recorded as the measure of PTC.

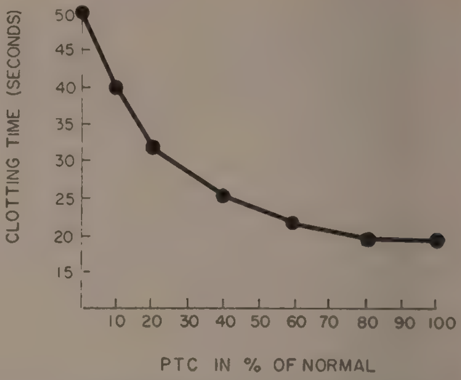

FIG. 1. Standardization curve of PTC assay. The assay is standardized by supplying PTC in graded amounts in the form of serially diluted normal serum.

A PTC standardization curve was prepared for the comparison of PTC content of unknown and normal sera. This was accomplished by supplying PTC to the assay in graded amounts in the form of normal serum, serially diluted. The clotting time at each dilution was plotted in per cent of normal (Fig. 1). Such a curve must be prepared for each new set of reagents. By means of the curve, the clotting times of unknown sera can be interpreted in per cent of normal PTC activity.

Results. The activity of PTC in coumarin-treated and vit. K-deficient patients was studied by means of the PTC assay. The well known deficiency of prothrombin and stable factor which these patients develop was measured by the one-stage prothrombin

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† Acacia was later found to be unnecessary and was replaced by saline.

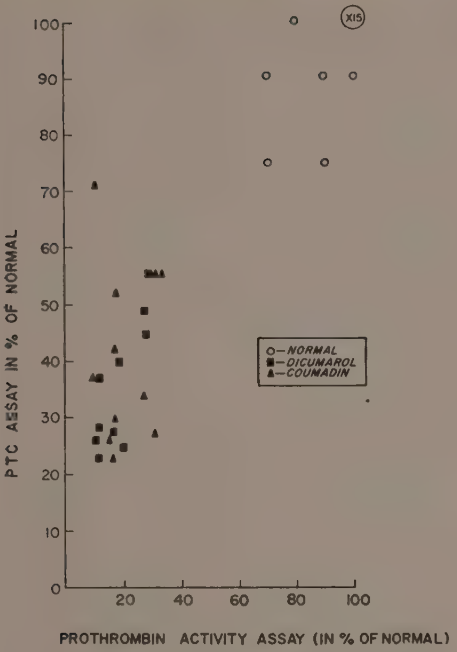

FIG. 2. Relationship between coumarin anticoagulation and PTC activity in 42 treated and untreated patients.

time test and the prothrombin time activity test. These latter assays are not influenced by variations in PTC, since the thromboplastin used in them is fully "activated" already.

Effect of 4-Hydroxy Coumarin Compounds on PTC Levels: Blood specimens were collected from 20 normal persons. A one-stage prothrombin activity test and PTC assay were done on each sample. These same studies were undertaken on 21 patients to whom either 3-3′-methylenebis (4-hydroxy coumarin), known as Dicumarol, or 3 (α-phenyl-β-acetylethyl)-4-hydroxy coumarin, known as Coumadin, had been administered for 4 or more days. These latter patients were suffering from either thrombophlebitis or myocardial infarctions. In Fig. 2 are recorded the test results obtained in both normal and treated patients. PTC levels are plotted against one-stage prothrombin activities. The Dicumarol-treated patients demonstrated significant deficiencies of PTC as well as a depression of the prothrombin activity test. Only one Coumadin-treated patient failed to demonstrate a severe deficiency of PTC. In a second group of patients there was an opportunity to study clotting factor levels before as well as during Dicumarol administration. Representative data, collected from a patient with thrombophlebitis, are shown in Fig. 3. A progressive depression of one-stage prothrombin activity, reflecting drug effect, was accompanied by a similar depression of PTC activity.

Effect of Vit. K_1 on a Depression of PTC Induced by Dicumarol: In patients treated with Dicumarol an attempt is made to maintain the one-stage prothrombin activity between 18% and 30% of normal (27-20 sec.) In one such patient, the drug dosage was inadvertently increased over a 3-day period so that the prothrombin activity decreased to 9% of normal and PTC activity to 34% of normal. At this time, 50 mg of vit. K_1 was given intravenously as a corrective measure. Six hours later, the prothrombin activity had risen to 28% of normal and the PTC activity

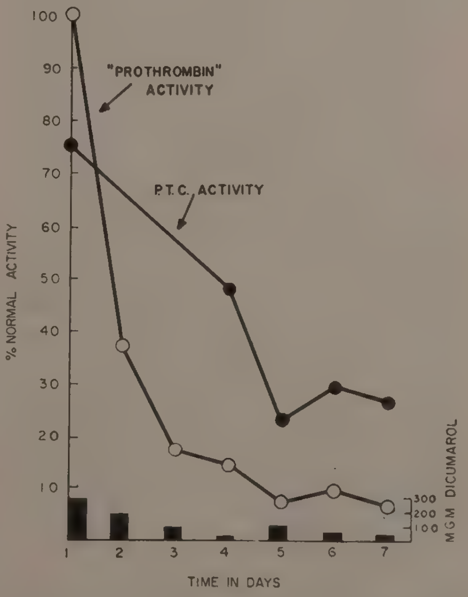

FIG. 3. Effect of Dicumarol on PTC levels and on the one-stage prothrombin activity test in a single patient.

to 43% of normal. After 18 hours, the results were 45% and 57% respectively.

Fat Maladsorption Syndrome: A child, aged 2 years, was found to be unable to adsorb fat-soluble vitamin K. This patient had cystic fibrosis of the pancreas. When tested she demonstrated a marked prolongation of the one-stage prothrombin time (58 sec., or 7% of normal) and a marked deficiency of PTC (8% of normal). Then 15 mg of menadione sodium bisulfite were administered intramuscularly. One hour later, the clotting factor activities had not changed. Eighteen hours after the vitamin administration, the prothrombin time was 18 seconds (57% of normal) and the PTC activity was 65% of normal.

Discussion. Deficiencies of prothrombin, fibrinogen, labile factor and stable factor can be congenital in origin; they can also reflect a vitamin deficiency or be manifestations of hepatic injury. In this regard, plasma thromboplastin component(PTC) is not different. Both vit. K and its coumarin antagonists exert a measurable influence on the activity of PTC, comparable to their effect on prothrombin and stable factor. This influence has been previously investigated, but with the exception of Sise *et al.*(5) the technics employed were too insensitive to demonstrate the moderate alterations that occur(1,13,14).

It is clear that stable factor and PTC have many common properties and characteristics. The one-stage prothrombin time test seems to present the strongest evidence for regarding these two factors as distinct. This test detects deficiencies in prothrombin, stable factor, labile factor and fibrinogen but not in PTC. The rabbit brain used as thromboplastin supplies a PTC-like activity artificially. However, human platelets when used as thromboplastin do not supply PTC-like activity. The necessary "activation" of human platelets by PTC and the other hemophilioid factors makes assay of these factors possible. With further investigation, PTC may prove to be as biologically important as are prothrombin and stable factor.

Summary. Deficiencies of plasma thromboplastin component (PTC) are not necessarily congenital in origin. Serum levels of PTC were depressed following the administration of two 4-hydroxy coumarin compounds to 24 patients. PTC activity was also reduced in a patient who developed a deficiency of vitamin K subsequent to an inability to adsorb fats (cystic fibrosis of the pancreas). In both groups of patients, PTC activity rapidly increased towards normal following the administration of vit. K analogues.

Grateful acknowledgement is made to Doctor Robert W. Coon for his continued interest and advice during this study.

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Lipemia and Glomerular Lesions in Rats Fed Diets Containing N-N′-Diacetyl- and 4, 4-4′, 4′-Tetramethylbenzidine. (22181)

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While testing a variety of chemical compounds for their ability to induce tumors in Buffalo-strain rats(1), it was observed that both N-N′-diacetylbenzidine and 4,4-4′,4′-tetramethylbenzidine caused severe lipemia accompanied by a characteristic renal lesion. The rats given these drugs died from the effects of the drugs before the time when induced tumors ordinarily appear following exposure to a carcinogen.

Methods: The benzidine compounds were dissolved in the propylene glycol portion of the diet incorporated at equal molar levels of 0.25 and 0.27% respectively into diet #222,[*] an otherwise excellent diet composed mostly of natural foodstuffs. This was fed to 5 groups of rats and 2 groups of inbred mice.

Results. No difference in the effects of the 2 compounds was observed. After 2 to 4 months on the regimen, the rats lost weight rapidly. During the terminal week of life, the loss often amounted to 20-50 g for individual rats. Fifty rats were autopsied. When killed during the terminal period, the blood was pale pink and clotted quickly. Generalized edema with ascites and hydrothorax was usually present and the kidneys were pale brown with slightly granular surfaces.

Frozen sections were made from the brain, liver, and kidney of 10 rats and stained by Sudan III. Large and small globules of bright red material, indicating the presence of fat were found in the vessels. This material was especially abundant in the glomerular capillaries (Fig. 1).

Tissues taken at autopsy were routinely fixed in Zenker-formol, embedded in paraffin, and stained with hematoxylin and eosin. Selected kidney sections were also treated by the periodic acid-Schiff and the Wilder reticulum method, Masson's trichrome stain, and toluidine blue.

Only an occasional glomerulus was altered in rats killed early, but changes were regularly found in the glomeruli of rats surviving 3-4 months (Fig. 2). The lesion was characterized by vacuoles within the glomerular loops. Bowman's space was often obscured, and there appeared to be adhesions between the layers of Bowman's capsule. The tubular cells were little altered, though many hyaline and waxy casts were present in the

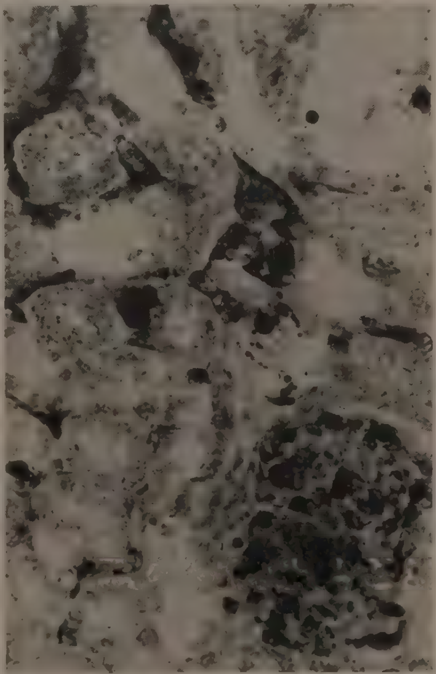

FIG. 1. Glomerular capillaries filled by lipid, which also appears within intertubular vessels. Sudan III. 280 ×.

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[*] Composition of diet No. 222: Commercial casein 3, skim milk powder 22.75, whole wheat meal (hard spring) 60.52, debittered brewer's yeast 1, whole liver powder (Wilson's) 1, NaCl 1.4, iron citrate .13, cod-liver oil 2, corn oil 5.2, propylene glycol 3.

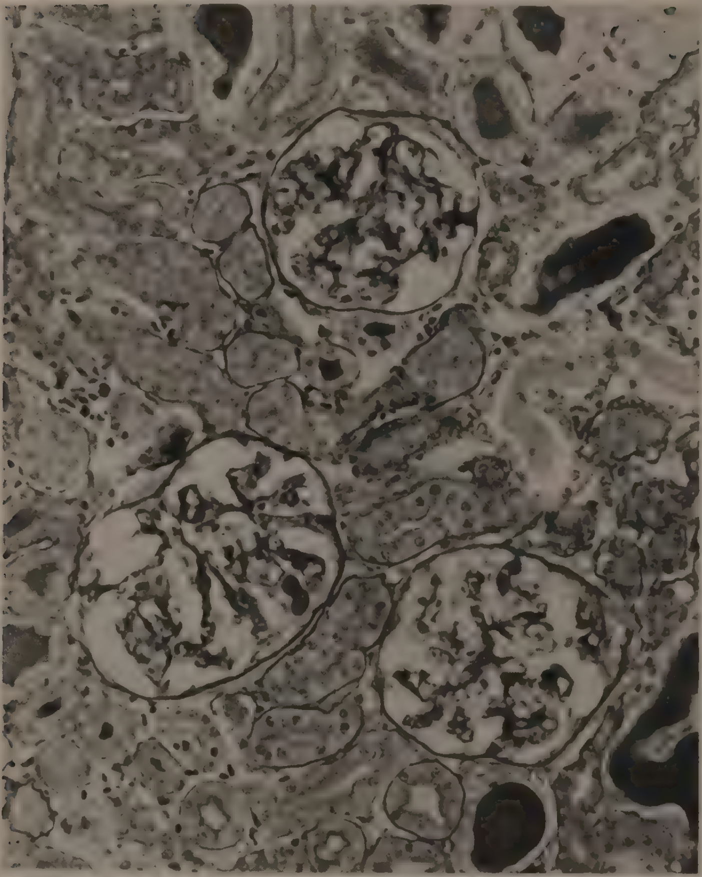

FIG. 2. Three glomeruli, showing small cystic dilatations, and obliteration of Bowman's space. Tubular cells appear normal, but heavily stained casts are present in the lumens. Periodic-acid Schiff. × 280.

tubular lumens. A large number of control animals examined showed none of the changes described.

The mice showed no effects from ingesting the drug; and only those lesions usually found in untreated mice of the strains tested, C3Hb/HeN and A, were observed at autopsy.

Discussion. The toxic effect of the 2 benzidine compounds at the doses given was so severe for rats that the animals failed to survive the period usually required for the induction of tumors. Consequently no appraisal can be made as to the carcinogenicity of these compounds.

Kidney lesions similar to this, to our knowledge, have never been observed in untreated rats. However, kidney lesions having many of the same morphologic features have been described by Wilens and Stumpf (2) in rabbits treated with cortisone. The initial and constant alteration of the rabbit kidney lesion was a moderate and fairly uniform dilation of almost all glomerular capillaries. The rabbits also had an extreme degree of lipemia, and Wilens and Stumpf suggested that the altered proportions of the blood lipids might cause protracted periods of glomerular capillary stasis. This effect of cortisone has not been found in other species, including man. The dilated capillaries noted in the rats receiving the drugs appeared to be empty after paraffin embedding, but were shown to be filled with fat in frozen section preparations. The small number of erythrocytes in the blood plasma in the rats and the filling of the lumens with fat may be a reason why the dilated capillaries appeared empty after paraffin embedding.

Summary. Lipemia and a glomerular lesion consisting of many fat filled spaces in the glomerular tuft appeared in from 2-4.5 months in Buffalo-strain rats fed diets containing N-N′-diacetylbenzidine and 4,4-4′, 4′-tetramethylbenzidine. No similar lesions were found in two strains of mice ingesting diets containing these two chemicals.

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Effect of Changes in Environmental Temperature on Stomach Emptying in Rats.* (22182)

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Rats transferred from room temperature to cold environment lose weight during the first few days. One obvious reason seems to be that, in spite of the increased caloric requirements in cold, the animals do not consume more food during this time than they did at room temperature. In search for the cause of this unsatisfactory food intake in experiments reported below we investigated specifically the question whether gastric emptying time is influenced by changes of environmental temperature. Although it has been shown earlier (1) that stimuli for food intake do not originate from the gastro-intestinal tract, everyday experience indicates that disturbances in physiological functions of the digestive organs may interfere with voluntary food intake. An early and important phase of food utilization is emptying of the stomach. We, therefore, considered first the changes in stomach emptying of animals exposed to lower temperature. The effect of changes in environmental temperature on stomach emptying has been discussed earlier(2) but the conclusions were equivocal. We decided, therefore, to investigate this problem directly applying a method for determination of stomach emptying time which proved to be valuable in our earlier investigations(3). The method is based on determination of N found remaining in the stomach 2 hours after voluntary consumption of a measured quantity of protein.

Methods. Male, Sprague-Dawley rats of 110-220 g weight were used. Stomach emptying time was determined by a method based on the method of Cori(4). However, we found, in preliminary experiments, that stomach emptying time varied widely after forced feeding by stomach tube. Excitement of the animals may be responsible for these variations. Consequently, adult male rats were trained to eat, spontaneously, definite quantities of protein after 24 hours starvation. Animals were maintained at room temperature of 22.2°-0.5°C. After 24 hour fast, with free access to water, each animal was presented with 2,-2,2 g of low fat tuna meat.† The N content of each batch of tuna meat was determined and the amount offered was calculated so that approximately 86 or 100 mg was consumed by the animal. Only those animals which consumed all of the tuna without spillage, within 15 minutes, were used for the experiments. After a digestion period of 2

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* These investigations have been supported by Contract USAF Arctic Aeromedical Laboratory.

† "Chicken of the Sea" brand Dietetic Tuna was used after draining of the cans.

hours, with free access to water, they were sacrificed by a blow on the head. The abdomen was opened immediately and ligatures were placed at the cardio-esophageal, gastroduodenal and ileo-cecal junctions. Stomach and small intestines with their contents intact were then removed, and after opening the lumen the contents were rinsed with distilled water into separate beakers. These washings were transferred to 100 ml volumetric flasks containing 10 ml concentrated sulfuric acid. The flasks were then placed in a boiling water bath for 90 minutes to completely disintegrate all proteinaceous material. After this digestion, samples were diluted with water to 100 ml volume. The N was determined on an aliquot part after further digestion with sulfuric acid, either by nesslerization using a Lumetron photocolorimeter, or by semi-micro Kjeldahl method. Both methods gave closely comparable results. The same procedure was followed with animals subjected to cold and heat with the following alterations: *For acute cold studies* immediately after all the tuna was eaten at room temperature, the cage with the animals was placed in a large walk-in refrigerator at 3°C for the 2-hour digestion period. *For acute heat studies,* immediately after all tuna was eaten at room temperature, the cage with the animals was placed in a dry heat room, temperature 37.8°C for the 2-hour absorption period. *For studies with cold adaptation* 5 days prior to start of experiment, animals were placed in the refrigerator at 3°C. They received food (Purina Chow) and water *ad lib*. for 5 days. The sixth day each animal was fasted 16 hours. This shorter fasting time was chosen to compensate for the increased caloric demand in the cold, and because we had found previously, that stomach and intestines were entirely emptied after 16 hours of fasting in the cold. After the 16-hour fast each animal was presented with tuna and remained at 3°C for 2 hours after consumption of food; they were then removed and sacrificed instantly. *Heat adaptation studies*, 5 days prior to start of experiment, animals were placed in a dry heat room with temperature 37.8°C. They received food (Purina Chow) and water *ad lib*. for 5 days. On sixth day animals, after 24-hour fast, were presented with tuna meat. They remained at 37.8°C for 2 hours after consumption of food and thereafter were removed and sacrificed instantly. In 3 animals stomach emptying was investigated after only one-day heat adaptation.

Results of our experiments are condensed in Table I. These data show that at normal room temperature, about 87% of the consumed N leaves the stomach within 2 hours. These values agree very well with our earlier findings. In animals exposed to *cold* after consumption of food, the amount of food left in the stomach after 2 hours was much larger. In the first 4 rats only about 63% and in the 8 rats belonging to another batch of animals, only 27% of the protein had left the stomach. We cannot account for the difference in the reaction of the 2 groups of cold exposed animals, but inspection of the data and statistical evaluation show that emptying of stomach was significantly delayed on acute exposure to cold in both groups. Consequently, we repeated the experiment with animals which were adapted to cold. The results in these rats showed that stomach emptying in animals adapted to cold was identical with that observed in normal rats at room temperature.

In the next group of experiments, animals were transferred to the hot room after consumption of food. Stomach emptying was even more delayed than after exposure to cold. In some cases the N content of stomach after 2 hours was actually higher than the amount consumed. This might indicate that a profuse gastric secretion was induced by heat, and the N content of this secretion was responsible for the increased amount of N found in the stomach(5). Further experiments on animals which had been acclimatized to heat for 5 days show that similar to adaptation to cold, adaptation to heat restores the stomach emptying to normal values. We do not know how fast adaptation occurs but the few experiments performed to date (Nos. 52-54) show that a 24-hour exposure to heat is not sufficient to restore stomach emptying time to a normal value.

To determine whether gastric digestion has

TABLE I. Influence of Environmental Temperature on Elimination of Dietary Protein from Rat Stomach.

		2 hr after food consumption		
Exp. condition	mg N consumed	mg N in stomach	mg N in intestines	Avg % of N emptied from stomach
At room temp.	86	6.7	14.5	
	"	6.4	14.7	
	"	5.3	14.2	
	"	10.3	14.4	91.8 ± 2.75
	100	8.7		
	"	16.3		
	"	18.6		
	"	14.		85.8 ± 3.86
Acute cold exposure	86	25.7	11.6	
	"	29.6	12.3	
	"	38.9	12.4	
	"	33.9	7.5	
	"	69.0		
	"	61.5		
	"	60.5		
	"	60.0		
	"	62.5		63. ± 6.48
	100	74.		
	"	83.		
	"	65.		
	"	69.		27. ± 4.12
After 5 days cold adaptation	86	15.9	14.6	
	"	14.0	18.2	
	"	5.0	15.3	
	"	10.1	12.2	
	100	21.3		
	"	7.3		
	"	24.5		
	"	15.0		
	"	26.7		84.9 ± 8.10
At room temp. after 5 day cold adaptation	86	12.6	12.4	
	"	14.6	18.6	
Acute heat exposure	86	55.0	13.0	
	"	57.0	16.8	
	"	68.0	17.0	
	"	54.0	12.2	
	"	67.0	11.4	30. ± 7.84
	100	100.0		
	"	116.0		
	"	131.5		
	"	132.5		
	"	133.5		0.
5 day heat adaptation	100	14.3	17.8	
	"	18.8	17.6	
	"	21.9	16.4	
	"	26.2	15.8	
	"	15.4		
	"	22.2		
	"	12.0		
	"	8.5		
	"	15.4		83. ± 5.68
After 24 hr heat adaptation	86	48.0	12.2	
	"	54.5	8.4	
	"	64.5	14.6	

been disturbed by acute changes of environmental temperature, we investigated the N present in intestinal contents 2 hours after food consumption. These experiments were based on earlier investigations in which we found that hard to digest proteins such as zein, raw eggwhite or certain soy protein preparations are emptied from the stomach at the same rate as easily digestible proteins. The intestines contained, however, much larger quantities of proteinaceous material 2 hours after feeding of zein, raw eggwhite or soy protein, than after consumption of casein or fish protein(3).

Table I shows that intestinal N content in cold and heat exposed animals was very similar to the value observed in rats at room temperature. These results seem to suggest that changes in gastric motility and not disturbed digestion may be primarily responsible for the delay in stomach emptying after acute temperature changes.

Discussion. The experiments reported above show that following a protein meal, sudden transfer of rats from room to either high or low environmental temperatures, prolongs the gastric emptying time. The question whether decreased motility, closure of the pylorus or disturbance in gastric digestion or a combination of these factors is responsible for this effect can not be definitely answered by our experiments. It seems, however, that changes of motility are the main cause. This conclusion is based on extensions of earlier experiments in which gastrointestinal propulsion of hard to digest proteins, such as zein, or raw eggwhite was studied. In those experiments stomach emptying was normal, but the intestines contained abnormally large quantities of undigested nitrogenous material (4). The finding that distribution of N between stomach and intestines of cold or heat exposed animals, is the same as that observed in control experiments, seems to indicate that disturbances of gastric digestion may not be the primary cause of the observed heat and cold effects. Our experiments indicate that after adaptation to changed environmental temperature, stomach emptying time is again similar to that observed at room temperature. We must, therefore, conclude that neither cold nor hot environmental temperature as such, but rather the sudden change of environmental temperature is responsible for the delayed stomach emptying. The common factor involved seems to be an increased epinephrine production which delays gastric emptying time. Epinephrine is produced, we found earlier, in abnormally large quantities during sudden cooling as well as sudden exposure to heat, but is not present in increased amounts after adaptation(6,7).

Summary. 1. Transfer of rats from room (22°C) to either low (3°C) or high (37°C) environmental temperature immediately following a protein meal delays emptying of stomach. 2. After adaptation to changed surrounding temperature stomach emptying was found to be again similar to that observed at room temperature.

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Effects of Intravenous Infusion of Experimental "Instant" Fat Emulsion Into Volunteer Subjects.* (22183)

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At the first *ad hoc* conference held by the National Research Council dealing with intravenous fat emulsions the stability and toxicity of such newly developed preparations were discussed(1). Except for variations in the type of fat (coconut oil, corn oil, sesame oil, etc.) and the character of the emulsifier ionic or nonionic, all investigators employed practically the same methods of preparation (2,3). The accepted procedure involved a high speed premix of constituents oil, emulsifier and water to form a crude blend after which followed mechanical pressure homogenization and finally sterilization by autoclaving. While these freshly prepared emulsions were well tolerated intravenously, administration of emulsions aged more than a month was correlated with a significant increase in reactions believed to be due to lipolysis, hydrolytic rancidity or physical alterations in particle size(1).

The present investigation was undertaken with a new type of intravenous fat emulsion prepared by means of special emulsifiers which make possible "instant" or spontaneous emulsification of oil and water mixtures for immediate intravenous administration. By this method the effects of aging on the emulsion with resultant toxic degradation products seemingly would be obviated. This study is concerned with the use of one such "instant" fat emulsion tested intravenously in experimental animals and in volunteer human subjects.

Method of study. The emulsion was prepared as follows. Fifteen g of sterile distilled whole coconut oil and 1 g of special emulsifier were placed in small intravenous bottles (250 ml). The emulsifier known as Drew #535 consisted of glycerol and polyglycerols partially esterified with mixed hydrogenated and unhydrogenated soy bean fatty acids. After addition of oil and emulsifier the bottles were capped with transfusion type rubber stoppers and autoclaved at 20 lb pressure for 35 minutes. After sterilization, these bottles were stored at room temperature. Before each infusion a bottle of the oil mixture was warmed and 10 ml of absolute alcohol was added under sterile conditions through the inflow hole. An almost clear solution formed upon mixing. Finally 125 ml of sterile 5% glucose in water was added to yield, after moderate shaking, an opaque emulsion. The latter was administered promptly by venoclysis through transfusion equipment containing a strainer-filter. The "instant" emulsion appeared uniformly white, flowed freely and remained stable for almost 5 hours. Occasional shaking prevented any tendency for separation. Caloric value approximated 230 calories for 150 ml. Microscopic examination with ocular micrometer under dark field illumination revealed active Brownian movement with 70% of the particles about 1 μ and the remainder up to 5 μ in size. The pH of emulsion was 7.35, refractive index 1.345, and dispersion a narrow spectrum band. The "instant" fat emulsion was first administered into a group of 10 dogs. Each animal received daily an infusion of 75 ml of the emulsion administered usually at the rate of 40 drops (2 ml) per minute. Observations were limited to temperature changes recorded at hourly intervals and to such visible reactions as vomiting, chills, etc. Following the animal studies, the same kind of emulsion was tested in a group of volunteer hospital patients. With each subject in the postabsorptive state, 150 ml of the emulsion was infused 20 to 30 drops (1 ml to 1.5 ml) per minute. The temperature, pulse, respirations and blood pressure were taken at hourly

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TABLE I. Reactions of Dogs and Human Subjects to Intravenous Infusion of "Instant" Fat Emulsion. 15 infusions in 10 dogs; 148 infusions in 46 humans.

Reactions	Animal %	Humans %
Minimum temp. 0-1°F	43	63
Moderate " 1-2°F	33	32
Maximum " 2°F	20	5
Vomiting	26	8
Chills	13	6
Diarrhea	20	2
Excessive salivation	13	—
Headache	—	9
Dyspnea	7	2
Sweating	—	6
Back pain	—	10
Chest pain	—	1

intervals for the first 12 postinfusion hours of a 24-hour study. Complete blood counts, sedimentation rates, hematocrits and standard urinalyses were performed by the usual technics at regular intervals. In several subjects lipid chemistry of blood was determined quantitatively for rate of clearance of fat from the circulation(4).

Results. Five dogs received single infusions of 50-75 ml; while 5 other dogs received 100 ml emulsion divided into 2 daily doses. The results have been recorded in Table I.

A total of 148 intravenous infusions of the fat emulsions were administered into human subjects. In this group of 46 volunteers, 28 received single infusions while 3 or more infusions were given to other 18 volunteers. The temperature changes associated with these injections and other reactions have also been incorporated in Table I.

Temperature elevations following animal and human infusions were always of a temporary nature and subsided rapidly to preinfusion level. Where multi infusions were given, temperature response varied from maximum elevation to moderate or minimal reaction with each infusion test. Constitutional reactions, when considered singly, totalled 44% but actually averaged 28% because in many cases these untoward effects were multiple in character; *viz.* vomiting, chills, etc. Changes in blood pressure during infusion and in the postinfusion phase were variable and showed no significant trend. Red blood cell counts, hematocrit cell volumes and sedimentation rates did not deviate markedly from normal. In 40% of white blood counts, the first postinfusion blood sample indicated a leukocytosis which averaged 19000 white blood cells after which there was a rapid return to preinfusion level which averaged 10200 white blood cells. Chylomicron counts increased markedly during infusion and then declined rapidly, usually within 4-5 hours after completion of infusion in a manner similar to the chylomicron pattern observed with other fat emulsions. Total blood lipids and partition fractions showed no characteristic trends (Table II). Postinfusion urine samples failed to reveal abnormal excretion of fat or other pathologic changes referable to the use of the emulsion.

Discussion. Considerable data have been reported on effects of "standard" type of fat emulsion but there has been no report with the present preparation. Our data have shown that infusions of the "standard" 10% fat emulsion in units of 1 liter were well tolerated in 77% of recipients when adminis-

TABLE II. Laboratory Data Pertaining to the Single Infusion of 125 ml of 15% "Instant" Fat Emulsion into Subject, A.S.

Infusion	Temp., F	Blood pressure, mm Hg	Pulse rate, min.	R.B.C. count (million)	W.B.C. count (thous.)	Hematocrit, %	Sedimentation time, mm/hr	Chylomicron count	Total lipids, g %	Phospholipids, mg/%	Total cholesterol, mg/%	Cholesterol esters, mg/%	Neutral fat, mg/%
Pre	98.8	140/88	86	4.26	9.8	46	28	4	.63	160	123	83	84
End of	99.0	160/88	98	4.24	11.0	40	36	108	.88	180	150	114	273
2 hr	102.8	160/90	140	4.41	11.2	40	36	86	.83	190	152	106	241
5 "	101.2	148/90	100	4.38	10.6	44	30	152	.75	190	157	110	123
7 "	100.6	145/90	94	4.29	10.6	44	34	58	.71	173	144	105	128
18 "	99.0	150/88	94	4.32	10.8	46	34	20	.79	165	152	106	196
24 "	99.0	154/80	90	4.38	10.2	48	20	6	.48	170	140	100	80

tered as a freshly made preparation but toleration declined to 69% when emulsions were 5 to 10 weeks old. A significant rise in concentration of free fatty acids and unstable peroxides or increase in size of fat particles up to 25 μ has been described as characteristic of aging emulsion. Accumulation of any of the latter products might be related to toxicity and thermogenicity of the emulsion when used for intravenous alimentation. These reactions may be compared to toxic effects produced experimentally by injection of free short chain fatty acids(5) or to pulmonary and pyrogenic effects associated with trapping of particulate matter in the lungs(6). Stability of an artificial emulsion should be considered to be a function of time during which physical alterations in size of fat particles and chemical interactions between water and fat may occur. A technic for rapid emulsification of oil and water mixtures with elimination of the factor of aging would therefore be warranted. Although the present "instant" fat emulsion cannot now be considered to be a suitable preparation for general clinical use, investigations along the lines described in this report have been continued.

Summary. An "instant" fat emulsion prepared by manual mixing of oil, emulsifier, alcohol and water was developed and tested. This type of emulsion obviated high pressure homogenization and was freshly prepared for each infusion. A total of 63% of the infusions were fairly well tolerated while in the other 37% reactions were observed.

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Modification of Tumor-Response to Rous Sarcoma Virus (RSV) by Hydrocortisone.* (22184)

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It is well known(1) that cortisone and hydrocortisone usually depress resistance of laboratory animals to viral infection and that effective doses of these steroids are frequently associated with evidence of increased multiplication of the virus. On the other hand, cortisone and several related steroids beneficially affect certain transplanted lymphosarcomas and leukemias in mice(2). However, rabbits injected with cortisone developed much larger tumors upon inoculation with rabbit fibroma virus although tumor size was not appreciably affected if injections of cortisone were delayed until appearance of the tumor(3). Data presented here show that (a) when Rous sarcoma virus (RSV) was inoculated into chicks pretreated with large doses of hydrocortisone, tumors appeared later and were greatly altered in appearance; (b) when hydrocortisone was discontinued the altered tumors promptly reverted to typical invasive type; and (c) when lower doses of hydrocortisone were employed or when injections of hydrocortisone were delayed until 2 days after in-

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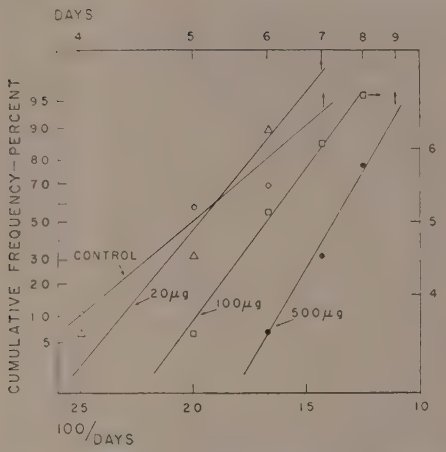

FIG. 1. Effect of pretreatment with hydrocortisone on latent period of RSV.

oculation of RSV the latent period was not affected; tumors which appeared were typically invasive but increased in size at a rate which was appreciably more rapid than that of control chicks.

Materials and methods. RSV (also referred to as chicken tumor I agent) was maintained by serial passage in chickens. A single lot (CT 581) of frozen standard RSV, prepared by differential centrifugation and preserved at –70°C as previously described (4), was used throughout. The diluent for standard RSV was saline containing 2% inactivated (56°C for 30 minutes) normal horse serum. Unsexed white Leghorn chicks under 7 days of age were used. Two tenths ml of suitably diluted RSV were inoculated into the subcutaneous tissue of left wing web. Ventral surface of the wing was swabbed with isopropanol and the hypodermic needle was inserted through the muscle into subcutaneous tissue of wing web, thus avoiding leakage. Each bird was examined daily by swabbing the wing with isopropanol and examining the wing with both direct and transmitted illumination. Hydrocortisone was suspended in the usual diluent(5) and 0.5 ml of the suspension were injected intramuscularly into the thigh muscle. Control birds received daily injections of diluent alone. Data were analyzed as previously described(6,7) using probit analysis(8).

Results. Fig. 1 shows effect of various doses of hydrocortisone on latent period of chicks inoculated into the wing web with RSV diluted 10^{-2}. Daily intramuscular injections of hydrocortisone were begun 2 days before inoculation of RSV and continued throughout the experiment. Control and treated groups consisted of 33 chicks each. It is clear that the latent period was prolonged in birds injected daily with 500 or 100 μg of hydrocortisone. However, the latent period in birds injected with 20 μg of hydrocortisone was not affected, but tumors which appeared were much larger than those in control birds.

In the above experiment the most prominent feature observed was the fact that all tumors which appeared in birds injected with 500 μg of hydrocortisone were grossly, markedly altered in appearance. In contrast to the usual rapidly growing, soft invasive tumor, the tumors which arose in hydrocortisone-treated birds were firm, sharply circumscribed and grew much more slowly. Characteristics of these altered tumors are summarized in Table I and photographs of typical and altered tumors are shown in Fig. 2-4.

Table II summarizes data showing the effect of daily dosage and dosage schedule on latent period and incidence of altered tumors. Altered tumors were produced only in chicks previously injected with large doses (500 μg/chick, *i.e.* 10 mg/kg) of hydrocortisone. Indeed, when dosage was decreased or delayed until after inoculation of RSV the tumors were not only typical and invasive but also were obviously larger and grew more rapidly

TABLE I. Characteristics of Tumors Produced by RSV* in Hydrocortisone-Treated and Control Chicks.

Characteristic	Control	Hydrocortisone-treated
Latent period†	4.6 days	7.6 days
Site of developing tumor	Wing web	Muscle
Type	Multiple	Single
Rate of growth	Rapid	Slow
Type of "	Diffuse & invasive	Spherical, sharply circumscribed
Consistency	Soft	Firm

* Standard RSV diluted 10^{-2}.

† LP_{50} or time in days to produce tumors in 50% of the chicks.

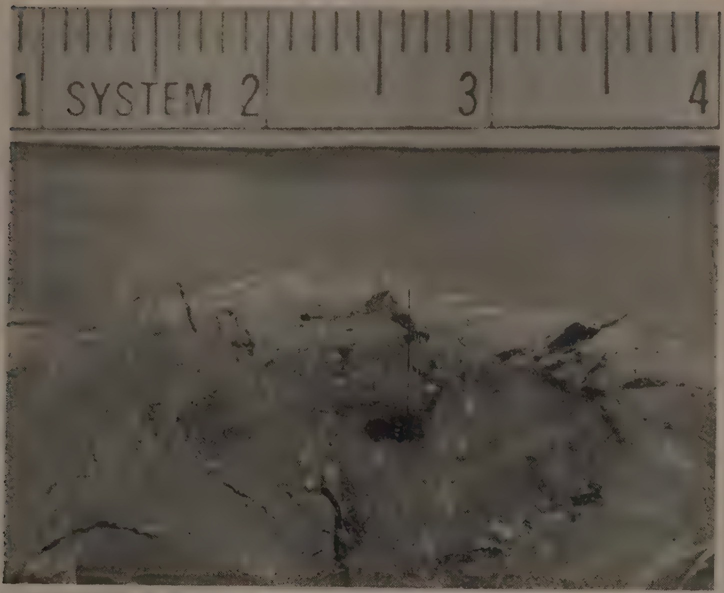

FIG. 2. Control tumor in wing. Twenty-one days after inoculation of RSV diluted 10^{-1}. Scale metric system.

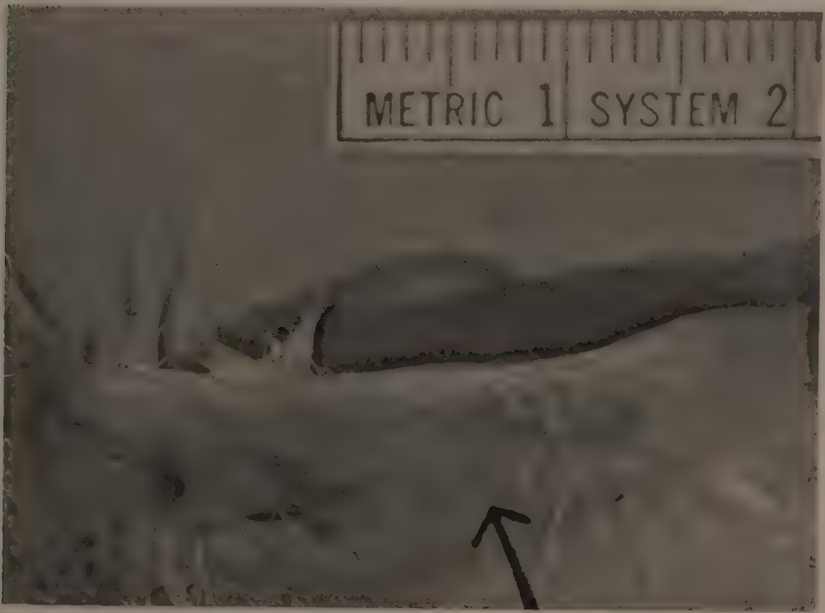

FIG. 3. Altered tumor produced by pretreatment with hydrocortisone. Front (ventral) view. Twenty-one days after inoculation of RSV diluted 10^{-1}.

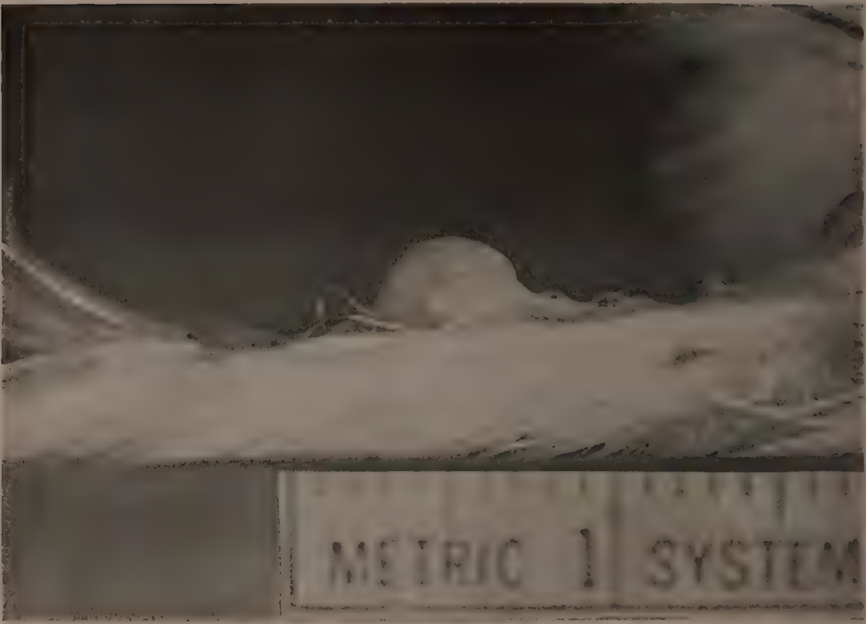

FIG. 4. Side view of altered tumor shown in Fig. 3.

than tumors in control birds.

Fig. 5 shows that when daily injections of hydrocortisone were discontinued almost all altered tumors promptly reverted to typical invasive tumors. In this experiment, 2 groups of 34 chicks each were injected daily with 500 μg of hydrocortisone and diluent, respectively, beginning 2 days before inoculation of RSV diluted 10^{-2}. All tumors in the treated group appeared as altered tumors and all control tumors were typical and invasive. On 10th day after inoculation of RSV the treated group was divided in half and injections of hydrocortisone were discontinued in one subgroup and continued in the other. It is clear (Fig. 5) that all but one of the altered tumors promptly reverted to typical invasive tumors in the subgroup in which hydrocortisone was discontinued and the tumors remained altered in those birds which continued to be injected with hydrocortisone. The single bird with an altered tumor which failed to revert when

TABLE II. Modification of Tumor-Response to RSV by Hydrocortisone.

Daily intramusc. inj. of hydrocortisone: Begun on day:	Daily dosage, μg	No. of chicks	Type of tumor: Typical, %	Altered, %	Latent period LP_{50}, days
−2	Diluent	30	100	0	4.3
"	20	"	100	0	4.2
"	100	"	90	10	5.3
"	500	"	0	100	6.7
"	Diluent	35	100	0	4.6
+2	500	"	100	0	4.5
0	"	"	97	3	5.4
−2	"	"	0	100	7.6

LP_{50} = Time in days to produce tumors in 50% of birds.
−2 = 2 days before RSV; +2 = 2 days after RSV.
RSV diluted 10^{-2}.

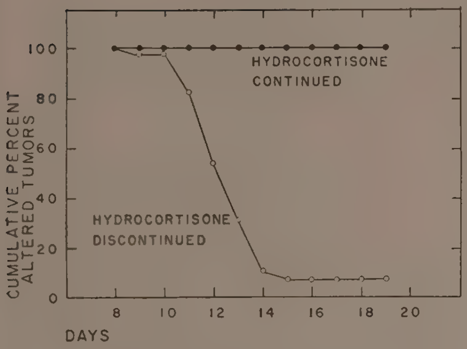

FIG. 5. Reversion of altered tumors after discontinuance of hydrocortisone.

hydrocortisone was discontinued, was also unusual in that the latent period of the altered tumor was unusually prolonged (10 days) by hydrocortisone.

In the single experiment described below the virus content of tumors altered by hydrocortisone was compared with that of control tumors. Groups of 30 chicks each were injected daily with 500 μg of hydrocortisone and diluent alone beginning 2 days before inoculation of RSV diluted 10^{-1}. Eleven days after inoculation of RSV, 6 birds from the control group and 11 from the treated groups were sacrificed and their tumors were removed aseptically, pooled, and stored in glass sealed tubes at –70°C. Potencies of the 2 pools of tumor tissue were compared with one another and with standard virus as previously described(9). The data indicated that the virus content of altered tumor tissue was lower than that of the control (typical) tumor tissue by a factor of approximately 10.

Discussion. Tumor-response to RSV was strikingly different in chicks that were pretreated with hydrocortisone. This modifying effect of hydrocortisone contrasts sharply with the increased rate of tumor growth observed when injections of hydrocortisone were begun after inoculation of RSV. Alteration of tumor-response to RSV by pretreatment of chicks with hydrocortisone is markedly affected by dosage of hydrocortisone employed. Thus, altered tumors were produced in chicks pretreated with large doses of hydrocortisone (10 mg/kg) while pretreatment with low doses (0.4 mg/kg) of hydrocortisone not only did not produce altered tumors but the typical, invasive tumors which arose were substantially larger than the control tumors. Studies on the mechanisms involved in these paradoxical effects are in progress.

Summary. When RSV was inoculated into chicks pretreated with large doses of hydrocortisone the tumors appeared later and were greatly altered in appearance. These altered tumors were sharply circumscribed, firm, whitish and grew more slowly than control tumors. When hydrocortisone was discontinued the altered tumors promptly reverted to typically soft and invasive tumors. When lower doses of hydrocortisone were employed or when injections of hydrocortisone were begun after inoculation of RSV the tumors which appeared were typically invasive and grew more rapidly than the controls.

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Acceptance of Tumor Homografts by Mice Injected with Antiserum. I. Activity of Serum Fractions.*† (22185)

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With certain combinations of transplantable tumors and inbred strains of mice, a normally resistant host can be induced to accept a tumor homograft if it is pretreated with preparations of mouse tissues prior to tumor grafting(1-3). Tumor homografts will also survive in mice injected with *antiserums* to mouse tissues, produced in either rabbits or mice(4-6).

Since the effect of these antiserums is opposite to that expected from a passive transfer of antibody, namely, *inhibition* of tumor growth, the study to be reported in the present paper was conducted to determine whether the serums had the chemical characteristics usually associated with antibodies, or whether they resembled the active substances in the tissues used to induce homograft acceptance. To this end, the serums were subjected to salt fractionation or zone electrophoresis, and the various fractions were tested for their ability to effect the survival of tumor homografts. The effects of heat and of sodium periodate on the activity of the serums were also determined, since it had been shown(2,3,7) that such treatments will destroy the ability of tissue preparations to abrogate host resistance.

Materials and methods. Antiserums were produced in rabbits or in C57BL/6Ks (C57 Black) mice. The rabbits were immunized with saline homogenates of freshly secured spleens from strain A mice. The first injection, which was made subcutaneously, consisted of 250 mg of spleen in 1 ml of Freund's adjuvant, containing 3.3 mg of heat-killed BCG. One week later, a series of intraperitoneal injections with saline homogenates of spleen (100 mg/ml) was started. Injections were given every third day, for a total of 5 injections. The amounts per injection were, respectively: 1.0, 1.5, 2.0, 2.0, and 2.5 ml. Bleeding for serum was done from the ear artery, by a method described by Cohen(8). The serums were stored in the deep-freeze, until needed. These serums, and those produced in mice (described below), exhibited hemagglutinating activity when tested with red blood cells of strain A mice.

Mice were immunized with an extract of a transplantable tumor (Sarcoma I), indigenous to strain A mice. The extract was prepared by homogenizing previously frozen tumor with an approximately equal weight of sterile 0.85% NaCl in a high speed blendor. The homogenate was centrifuged at 8500 x g for one-half hour, at room temperature. The supernatant was then diluted 1:10 with sterile 0.85% NaCl, and the diluted material was used for immunization. The diluted supernatant was stored at –26°C. The mice received a series of 5 intraperitoneal injections, 0.5 ml per injection, over a period of 2½ weeks. Twelve days after the last injection, the mice were bled by incision of the lateral tail vein and the serum was separated from the blood clot. In addition to these methods, the antiserum used for zone electrophoresis was produced in mice (C57BL/6Ks strain) inoculated subcutaneously with a tumor homograft (Sarcoma I). The mice were bled from the lateral tail vein 10 days after inoculation. The serum was separated from the clotted blood, freeze-dried in ampoules, and sealed *in vacuo*. Zone electrophoresis was carried out for us by Dr. Peter Bernfeld,‡ by a method described by Bernfeld and Nisselbaum(9). Five globulin fractions, thus prepared and freeze-dried, were reconstituted by

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† With the technical assistance of Bradley F. Bryant.

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TABLE I. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Antiserum Fractions Precipitated by Ammonium Sulphate.

Fraction injected				
$(NH_4)_2SO_4$ conc., %	Material ppt.	Amt inj. per mouse	No. of mice dying with tumors*	
	Antiserums produced in rabbits			
50–75	Albumin	25 mg	3/5	
”	”	15	0/5	
”	”	5	0/5	
33–50	α, β-globulin	5	5/5	
0–33	γ -globulin	5	5/5	
”	”	3	4/5	
”	”	1	5/5	
—	Unfractionated serum	.5 ml	6/6	
	Antiserums produced in C57BL/6Ks mice		Exp. 1	Exp. 2
50–75	Albumin	10 mg	0/5	1/ 5
33–50	α, β-globulin	5	4/5	5/ 5
”	”	2	—	5/ 5
”	”	1	1/5	—
”	”	.5	—	4/ 5
0–33	γ-globulin	5	5/5	—
”	”	2	4/5	10/10
”	”	.5	1/5	3/ 5
—	Unfractionated serum	1 ml	15/15	

* Numerators give the No. of mice dying with tumors; denominators, the total No. of mice in each group.

us with sterile 0.85% NaCl and dialyzed against 0.85% NaCl in autoclaved Visking cellulose sacks for 18 hours, at 4°C, to remove excess salt (since the fractions had been freeze-dried from a large volume of solution). The contents of the sacks were removed and diluted with sterile 0.85% NaCl to the concentration desired for injection into the mice. Salt fractionation of the serum proteins was carried out with a saturated solution of ammonium sulfate which was added dropwise to 20 to 30 ml of serum, maintained at a low temperature in an ice bath. The proteins precipitating in the salt concentrations of 0-33, 33-50, and 50-75% saturation respectively, were collected. Each fraction was then dissolved in distilled water, reprecipitated at least twice more, dialyzed against distilled water in a refrigerator, and freeze-dried. For injection, the freeze-dried material was reconstituted with sterile 0.85% NaCl to the desired concentrations. Treatment with sodium periodate was carried out on the globulin fractions of an antiserum that had been produced in mice. The globulins had been prepared by salt fractionation, as described above. The globulins, in a final concentration of 3.9 mg/ml, were mixed with $NaIO_4$ dissolved in 0.85% NaCl, and buffered with phosphate to pH 7.0. The final concentration of $NaIO_4$ was either 0.01 M, or 0.001 M, and the final concentration of buffer was 0.03 M. The mixtures were kept for one hour at a temperature of approximateely 21°C. An equal volume of glucose solution (10 mg/ml) was then added to inactivate the remaining $NaIO_4$. The measure of biological activity of treated serums and serum fractions was their ability to effect survival of tumor homografts. The various preparations were injected intraperitoneally into the prospective hosts several hours before grafting of the tumor. The transplantable mouse tumor used as the test homograft was the strain A tumor Sarcoma I (SaI). This tumor grows in 100% of A/Ks mice, killing the animals within 3 to 5 weeks after inoculation. Hosts were male mice (except for one experiment in which females were used to test heated serums) of the C57BL/6Ks strain, which is genetically unrelated to the A strain. SaI rarely grows in an untreated C57BL/6Ks mouse. All mice were 2 to 4 months old at the start of the experiments. The grafts were placed subcutaneously, by trocar, in the suprascapular region. Growth of the grafts was followed by means of per-

TABLE II. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Antiserum Fractions Prepared by Zone Electrophoresis.*

Material inj., 3 mg/mouse		No. of mice dying with tumors
α-globulin	Fraction II	0/5
Intermediate fraction	III	”
β-globulins	IV	”
Main β-globulin	V	1/5
γ-globulin	VI	5/5

* Antiserum prepared in C57BL/6Ks mice.

iodic palpation, until the mice either died with a progressively growing tumor, or remained without a sign of growth for at least 2 months, at which time they were killed and classified as "negative".

Results. The 2 serum fractions precipitated by 0-33% saturated $(NH_4)_2SO_4$ (mainly gamma-globulin) and 33-50% saturated $(NH_4)_2SO_4$ (mainly alpha and beta-globulin) appeared to be equally active (Table I). The fraction precipitated by 50-75% saturated $(NH_4)_2SO_4$ (mainly albumin) was almost completely inactive. That the activity of the globulin fractions was not due to some possible alteration in the proteins during salt fractionation, was demonstrated by the lack of activity of fractions similarly obtained from normal serum, derived from C57BL/6Ks mice.

The fractions obtained by zone electrophoresis can be assumed to have a degree of purity which cannot be achieved by salt fractionation. Fraction VI (practically pure gamma-globulin) was fully active, while a slight activity was displayed by the neighboring fraction V (the main beta-globulin) (Table II). Fractions II, III, and IV, which respectively are alpha-globulin, an intermediate fraction between alpha and beta-globulin, and lastly mostly beta-globulin (actually 2 beta-globulins), did not effect homograft survival. One point of interest is the observation that the homografts in the mice that had received fraction VI showed a marked initial growth (as compared with uninjected controls or with the animals receiving the other fractions). This was followed by a marked regression and then a resumption of growth, with eventual death of the host. The possible significance of this is considered below in the discussion.

The results obtained with the heated serums (Table III) show that the antiserums produced in both rabbits and mice are fully stable when heated to 56°C for one hour. Whole rabbit antiserum heated to 70°C for one hour retained its activity, though there was a slight impairment, as shown by a slower initial growth rate of the tumors. In the case of the antiserums produced in mice, heating to 70°C inactivated the whole serum to a large extent, though not completely, but did not impair the activity of the globulin fractions (produced by salt fractionation).

Sodium periodate did not markedly alter the effectiveness of the antiserum (Table III). There was a slight indication of impairment at the higher concentration (0.01 M $NaIO_4$), in terms of slower growth of the homografts in the mice receiving the serum treated at this level of sodium periodate. This, however, is in contrast to the complete destruction of the activity of tissue treated with $NaIO_4$(7). Though there was a lower number of "takes" in the mice receiving the dextrose-serum mixture, this is not considered significant, since only 4 of the 5 mice which received untreated

TABLE III. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Heated Antiserum, or Antiserum Treated with Sodium Periodate.

Material inj. and treatment	Amt inj. per mouse	No. fatal /No. inj.†
Antiserum produced in rabbits		
Whole serum, 100°C/20 min.	1 ml	0/ 5‡
70 / 1 hr	1	5/ 5‡
70 / 1	.5	4/ 5
56 / 1	1	14/14§
Antiserum produced in C57BL/6Ks mice		
α, β-globulins,* 70°C/1 hr	2 mg	3/ 5
γ -globulin,* 70 /1	2	5/ 5
Whole serum, 100 /1	1 ml	0/ 5
70 /1	1	2/ 5
56 /1	1	5/ 5
.01 M $NaIO_4$	3.9 mg	4/ 5
.001M $NaIO_4$	3.9	5/ 5
Dextrose	3.9	2/ 5
Untreated	1.0 ml	4/ 5
Dextrose	1.0	15/15

* Prepared by fractionation with ammonium sulphate.

† Males used throughout, except as indicated.

‡ Females.

§ 9 females, 5 males.

serum, in this experiment, grew the homografts. Furthermore, in another experiment, in which dextrose was added to the serum in even larger quantity, the effectiveness of the serum was not diminished.

Discussion. These data, together with others to be published later, indicate that the substance in the antiserums which is responsible for the survival of the tumor homografts was not introduced by the injection of the tissue extracts used for immunization, but is an antibody component (or components). This conclusion is supported by the observation that the antiserums, unlike tissue preparations, were not inactivated by sodium periodate, and that tissue preparations are labile at temperatures above 60°C(2,3), while the serums are comparatively stable at 70°C. The partial loss of activity of the whole serums heated to 70°C, as compared with the full retention of activity of the globulin fractions similarly treated, is in accord with previously reported observations(10) that isolated protein fractions are more stable to heat than the unisolated proteins in whole serum.

Although the results obtained with the fractions produced by zone electrophoresis would indicate that the activity is most likely associated with the gamma-globulin portion, it is possible that at higher dosage levels than those we used, activity might have been demonstrated by some of the fractions other than fraction VI (gamma-globulin). The limitation on the dosages used was determined by the amount of material on hand, and the inaccessibility of the equipment used for fractionating the serums. The possible importance of dosage is emphasized by the comparatively slow rate of growth of the homografts in the mice pretreated with fraction VI. The lower activity of this material, as compared with the gamma-globulins produced by salt fractionation, may be attributed to a lower potency of the antiserum used for fractionation. This antiserum had been taken from mice bled 10 days after inoculation with the immunizing tumor, and it is known (data to be published) that the peak potency of the antiserums is first reached at between 14 to 21 days.

Summary. Antiserums to mouse tissues, produced in rabbits or mice, induce acceptance of an otherwise incompatible tumor homograft in mice, when injected into the host prior to grafting of the tumor. Activity of antiserums was associated with the globulin fractions, and was most concentrated in the gamma-globulin fraction. The activity was not impaired by treatment with sodium periodate, or by heating to 56°C. It was slightly impaired by heating to 70°C, and was destroyed at 100°C.

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Tissue Weights of the Rat. I. Normal Values Determined By Dissection and Chemical Methods. (22186)

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Change in body weight, or more particularly tissue weights, is an important and physiologically significant datum in many experimental situations. This paper will describe practical methods for obtaining and expressing tissue weights required for this type of study. These procedures result from experience gained in quantitative dissection of some 300 rats (2-3000 tissue weights) in the course of a 4-year study of alterations in tissue metabolism associated with radiation sickness. They are designed to provide a statistically stable base-line for estimating changes in weight of each major tissue component of the body. The weight of such organs as liver, kidneys, spleen, testes, etc., can easily be obtained by direct dissection, and thus require little special discussion. Unfortunately their total weight constitutes only a minor fraction of total body weight and frequently contributes in a trivial way to a total body weight change. The major portion of body weight lies in the musculoskeletal system. Because of extensive interconenction between muscle, skeleton, marrow and connective tissue, this fraction also presents one of the greatest problems in quantitative separation of different components. For purposes of description, we have arbitrarily divided the musculoskeletal system into 2 major parts: (a) total muscle mass, and (b) non-muscle components which are grouped under the general heading of supportive tissue. A more complete description of the various components of these two major parts will be given as we proceed.

The method for determining how much of the total musculoskeletal weight can be ascribed to each of these two categories depends upon the 2 observations that: (a) some 70% of the muscle mass of the rat can be removed from the supportive tissue in about 20 minutes, and (b) there are sufficiently large differences between the chemical compositions of supportive tissue and muscle so that it is possible, by direct chemical means, to estimate the amount of muscle still adhering to the supportive tissue at the end of the partial dissection. Chief among the chemical differences between these tissues are the differences in per cent water, per cent ash, and the differences in actomyosin, desoxyribonucleic acid and creatine concentrations. This approach has not only the advantages of speed and ease, but also yields such information as the fresh weight (and per cent water) of supportive tissue fractions and the weight of the total marrow mass which is difficult to obtain by any other method. For further discussions this partially dissected sample, consisting on a fresh weight basis of nearly equal weights of muscle and supportive tissue, will be termed the "stripped carcass." The ideal substance to use in estimating the relative weights of muscle and supportive tissue in the stripped carcass, would be one which is constant in all muscle, absent in skeleton and connective tissue, and is easily and accurately determinable. Empirically, the determination of actomyosin or the per cent of ash have been found most useful for this purpose. Where other analyses are also desired on this same sample the ash procedure is the more convenient. This combination dissection-chemical approach was also used in estimating the total amount of marrow in the skeleton. The useful chemical data in this case were the desoxyribonucleic acid and hemin contents.

Chemical methodology. The reagents and general pattern of procedure for determining actomyosin are those described by Benson, Hallaway and Freier(1). Quickly dissect, weigh and freeze the sample for storage. Chop the frozen sample, place in a water cooled Waring blendor cup with 3½ ml of Webers solution (0.6M KCl in bicarbonate buffer) per gram of sample, and blend 8 minutes at reduced speed (furnish 40-60 volts AC to the Waring blendor motor). Wash into a beaker

TABLE I. Tissue Weights Expressed in Different Ways.

Tissue	As % of body wt	As % of Wc*		Data of Column 2 expressed on a:	
		Mean	σ	Fat-free basis	Dry, fat-free basis
GI content†	5.85	—	—	—	—
Depot fat‡	7.08	—	—	—	—
Muscle	45.5	52.3	1.5	52.9	42.1
Supportive	7.68	8.82	.52	8.98	19.24
Skin	18.0	20.7	1.6	19.2	26.6
Liver	4.15	4.77	.53	4.96	4.39
Lung	.56	.64	.10	.66	.43
Heart	.289	.332	.030	.345	.238
Kidneys	.76	.87	.15	.89	.69
Testes	.94	1.08	.13	1.13	.45
Spleen	.213	.245	.034	.256	.187
Brain	.550	.632	.046	.614	.300
Stomach	.502	.577	.038	.581	.435
Small intestine	1.93	2.22	.27	2.21	1.53
Large intestine	.89	1.02	.11	1.00	.63
Thymus	.125	.143	.025	.146	.095
Blood§	4.95	5.68	—	6.04	3.88

* Body wt minus wt of the gastrointestinal contents and depot fat.

† Fresh wt of material in gastrointestinal tract (on *ad lib.* diet of Purina Chow).

‡ Largely adipose tissue, though a small amount of miscellaneous tissue (bladder, etc.) was included.

§ Including fluid loss during dissection (by difference). For further details on blood volume and distribution see Caster, Simon, and Armstrong(8).

with an equal volume of 0.6M KCl, add 10 μl of 10% ATP per gram of stripped carcass, cover, and allow to stand in a refrigerator for 24 hours. Stir vigorously, transfer 20 ml aliquot(s) into centrifuge tube(s), add 20 ml of 0.6M KCl, centrifuge at 600 g for 10 minutes, transfer supernatant and recentrifuge for 15 minutes. Measure the viscosity of the supernatant before and after the addition of 20 μl of 5% ATP. The viscosity decrease observed for the stripped carcass is compared with that found for a sample of thigh muscle from the same animal, and the final result expressed as the per cent muscle estimated to be present in the stripped carcass (about 50%). The use of the per cent ash in the stripped carcass as a basis for estimation depends upon the fact that muscle contains only 4.58 ± .12% ash (on a dry, defatted basis) while the total supportive tissue was found in a separate series of dissections to contain 53.6 ± .7% (on a dry, defatted basis). The fresh weight figures could be used, but by expressing results on a dry-weight basis one eliminates errors due to evaporation loss and minimizes those resulting from physiological changes in the degree of hydration of the connective tissue. From these data it follows that for mixtures of muscle and supportive tissue the relation between composition and observed ash can be expressed as $X = 4.58 + .4898Y$, where $X = \%$ ash observed in the dry, defatted stripped carcass, and $Y = \%$ supportive tissue in the sample (expressed on a dry, fat-free basis). Desoxyribonucleic acid and hemin were determined in freshly extruded femoral marrow and in aliquots of completely dissected skeleton which had been dried, defatted and powdered in a Wiley mill to pass a 40-mesh screen. The DNA was hydrolyzed and extracted with 5% trichloroacetic acid at 85°C for 30 minutes, and the desoxyribose determined colorimetrically with Dische reagent(2). Since high concentrations of bone mineral interfere with maximal color development, a series of standards were analyzed in the presence of bone mineral so as to allow correction for this factor. For the determination of skeletal hemin, other aliquots were extracted with 0.1N NaOH, and the iron porphyrin determined by the pyridine hemochromogen method of Remington (3). This procedure is somewhat easier and more desirable because no empirical corrections are required.

Manner of expressing tissue weights. In comparing and discussing the observed tissue weights of different animals, it would be convenient to express these data in relative rather than in absolute terms. The most usual way of doing this is to express tissue weight as a per cent of total body weight. However, the total body weight contains two portions which may vary considerably from rat to rat and may change very rapidly under stress conditions; *i.e.*, the weights of the (a) body fat, and (b) gastrointestinal contents. It was found empirically that much more stable estimates were obtainable when results were expressed on the basis of a body weight corrected for these variables. The symbol, Wc, will hereafter be used to designate body weight minus the weight of body fat and gastrointestinal contents. The rats used in this study were male white rats (Holtzman Co., Madison, Wis.), 110 ± 10 days of age, weighing 338 ± 20 g. They were received at a weight of 250 to 280 g and raised to the final weight on a diet of Purina Chow in a 25°C room. Dissections were carried out under ether anesthesia. The tissue weight data are summarized in Table I.

Procedure. Skin and eviscerate the anesthetized rat, and remove the depot fat from the carcass. Remove the brain, spinal cord, and any extraneous tissue. Weigh the musculoskeletal remainder and remove a sample of thigh muscle for the determination of ash weight. Divide the musculoskeletal system so that one person can strip the muscle from the head and shoulder girdle, while the other person removes the muscle from the other portions. About 70% of the muscle mass can thus be removed from the supportive tissue in 20-25 minutes. After a few dozen dissections this procedure became highly repeatable, yielding a stripped carcass sample which weighed 18.18 ± .60% of Wc. After weighing, the stripped carcass was either: (a) analyzed for actomyosin or (b) dried and ashed. As described above, either of these procedures provides an estimate of the residual muscle on the sample. The total supportive tissue weight in this sample was 8.82 ± .52% of Wc (22 rats) on a fresh weight basis.

Components of supportive tissue. A separate series of total dissections (22 rats) indicated that the weight of the skeleton (with teeth and marrow, but freed of connective tissue) was 6.86 ± .33% of Wc, and that the teeth weighed 0.184% of Wc. Analysis of these skeletons for DNA and hemin followed by a comparison of these results with the DNA and hemin contents of freshly extruded femoral marrow led to an estimated total marrow weight of 0.40% of Wc. The weight of the connective tissue (present in the stripped carcass but removed during the total dissection of the skeleton) was estimated by difference to be (8.82 — 6.86 =) 1.96% of Wc. It should be noted that this does not constitute the total connective tissue of the body, but only that closely associated with the skeleton. All of these data are summarized in Table II, together with analytical data on the weights of water and fat present in each. All data are expressed as grams of weight for the average rat in this study (body weight = 337.7 g and Wc = 294.0 g). The composition of connective tissue was obtained by subtracting the composition of its muscle and skeleton components from the observed composition of the stripped carcass. The dry, fat-free skeleton (complete with teeth and marrow) was 4.33% of Wc, and its ash weight was 2.63% of Wc. These figures are in good agreement with those reported by Savchuck and Armstrong(4) for the 304 g rat. Their information on the individual bones may therefore be considered as an extension of Table II.

TABLE II. Summary of Supportive Tissue Components in the 338 g Rat.

	Wt in g			
Tissue	Fat	H_2O	Fat-free dry wt	Fresh wt
Total	1.07	9.37	15.49	25.93
Skeleton*	.83	6.59	12.73	20.15
Connective	.24	2.78	2.76	5.78
Teeth	.00	.11	.43	.54
Marrow	.01	.09	.81	1.17

* Including teeth and marrow.

Muscle mass. Subtracting the weight of the supportive tissue from the original weight of the musculoskeletal system sample, the total weight of muscle mass is obtained. This method of calculation, again, minimizes er-

TABLE III. Weight of Muscle Groups as % of Total Muscle Mass.

Muscle group	Mean	σ
Thigh	19.18	1.25
Legs	4.48	2.34
Abdominal	16.96	2.37
Shoulder	8.43	1.69
Face and head	4.06	.58
Back	22.44	1.73
Residual	20.62	6.24
Fat	3.81	.44

rors due to evaporation loss during dissection. The components of the total muscle mass were studied in a separate series of dissections. Table III lists the relative weight of the major muscle groups which comprise this total muscle mass. All results are expressed on a fat-free wet basis and the total fat associated with these muscles is given separately at the bottom of the table. The thigh muscles were obtained by making an incision around the dorsal border of the ilium and extending it medially and ventrally along the os coxae, being careful to avoid the psoas major and psoas minor muscles and the quadratus lumborum. Another incision was made around the knee joint, and the muscles between the two incisions were removed. Then the muscles of the leg from the knee joint on down were removed. The muscles of the foot were not taken. These muscles taken from both legs constitute the leg muscles referred to in Table III. A ventral incision was made in the midline and another incision was made dorsally, just lateral to the transverse processes of the abdominal vertebrae and extended as far caudally as the tip of the ilium and as far cranially as the false ribs. The muscles lying between these two incisions were removed. The psoas major, psoas minor, iliacus, and quadratus lumborum were cut from their origins and insertions. All of these muscles taken together were included in the abdominal muscle group. An incision was made around the scapula and the muscles were stripped from the scapula, brachium and antibrachium. These are referred to as the shoulder muscles. The head was severed at the level of the epistropheus bone and the mandible was removed so as to allow access to the muscles of mastication and the buccinator muscle. The superficial muscles of the head and skull were then removed. This group is referred to as the face and head muscles. The ribs were disarticulated and the intercostal muscles removed. To these were added the major portion of the muscle which could be removed from the trunk. This is defined in Table III as the back muscles. There still remained a certain amount of muscle on the skeleton which corresponded roughly with that present in the stripped carcass sample, and it is here referred to as the residual muscle. This is mainly composed of the anterior and lateral vertebral muscles and the serratus posterior muscles which are difficult to remove from the vertebrae, together with the muscles of the manus and the foot. As seen in Table III the thigh muscles constitute one of the largest of these muscle masses and is, on a percentage basis, also one of the most constant. From its ready availability it furnishes one of the most common sources for muscle samples for chemical analysis.

Other tissues. The items in Table I include two miscellaneous classifications: "body fat" and "blood." The subcutaneous and circumrenal fat constitute by far the largest part of "body fat," but also included in this category are such small tissues as, bladder, pancreas, spinal cord, eye balls, etc. Obviously any of these tissues could be removed from this miscellaneous classification and measured separately if one so desired. The item termed "blood" is even less well defined. At the end of the dissection it is quite usual to find that the sum of the recorded tissue weights equals 94-96% of the weight of the anesthetized animal as measured prior to dissection. By visual inspection it is quite obvious that a certain amount of blood and other fluids have been lost in the course of the dissection. The best evidence(5-8) indicates that some ⅔ of the blood volume is present in the large vessels and can be lost in the course of this procedure. Since the magnitude of this estimate corresponds so well with the observed 4-6% weight loss, we have arbitrarily termed it "blood." In a series of 22 normal rat dissections, a $-.83$ correlation ($P<.01$) was observed between variations in "blood" weight and the variations in muscle mass weight when expressed as a per cent of Wc. We take this to indicate that another factor entering into this "blood" item is the loss of muscle water by evaporation during the course of the 20-25 minutes required to remove the major portion of the muscle mass. The constancy of the "blood" weight is therefore of interest as an index of the repeatability of the dissection procedure.

Results. Each figure in Table I represents the mean of 16 ± 5 independent dissections. In the first column, these data are expressed as a per cent of the total body weight of the intact rat. As indicated above, a more repeatable and usable result can be obtained if tissue weights are expressed as a per cent of Wc (body weight minus the weights of the contents of the gastrointestinal tract and the depot fat) as is done in the second column. In the third column are given the corresponding standard deviations. These help to indicate the magnitude of change in tissue weight resulting from stress conditions that one might expect to detect with confidence by this dissection-chemical method. When tissue weight comparisons are made on a completely fat-free and fat-free dry-weight basis the results seen in columns 4 and 5 are obtained.

Summary. A combined dissection and chemical approach to the problem of determining tissue weights has been described. It allows the exact determination of total muscle mass and supportive tissue weight in a fraction of the time required by the complete dissection approach. It also provides estimates of the total marrow weight and the fresh weight of the connective tissue, which are difficult to obtain in any other manner.

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Tissue Weights of the Rat. II. Changes Following 700 r Total Body X-Irradiation. (22187)

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In the first 4-6 days following total body x-irradiation with an LD_{50} dose of x-ray, the rat loses some 15% of its body weight. An exact description of this weight loss in terms of the weight changes of the individual tissues of the body is of considerable importance in providing a basis for any metabolic study of the nature of radiation damage.

Methods. Groups of 300-350 g male white rats (Holtzman Co., Madison, Wis.) were given 700 r of total body x-irradiation (140 kv, ¼ mm Cu, 1 mm Al) and sacrificed at one of a series of periods following irradiation. Unirradiated control animals for each of the different experimental periods were distributed within each group of experimental ani-

TABLE I. Tissue Weights following Total Body X-Irradiation with 700 r (Expressed as a % of Control(1)).

	Periods post x-ray								
Tissue	3 hr	12 hr	1 day	2 days	3 days	4 days	6 days	8 days	10 days
Muscle	96.7†	94.4†	96.2†	91.3†	88.3†	80.2†	85.3†	85.4†	87.6†
Supportive	99.2	103.1	103.4	113.1†	100.7	113.9†	105.8*	102.4	106.7*
Skin	97.0	97.4	101.4	93.8	86.4	91.1	87.7	94.3	98.2
Liver	101.3	95.8	96.4	90.8	97.2	94.7	94.6	96.1	106.5
Lung	110.8	90.2	101.9	99.4	99.4	112.6	97.0	94.7	97.3
Heart	101.5	105.4	104.8	103.0	97.3	98.8	98.8	97.3	106.6
Kidneys	106.2	104.6	99.0	100.9	86.4	92.1	98.5	94.2	103.3
Testes	99.7	115.0	105.6	108.3	103.1	107.3	88.4	90.2	101.5
Spleen	79.6†	64.2†	57.3†	47.5†	37.4†	36.7†	40.9†	43.1†	60.7†
Brain	103.8	101.6	105.7	107.4*	99.1	106.8*	104.0	97.2	102.1
Stomach	97.1	110.7*	107.5	103.0	106.4	101.1	104.4	86.4†	105.3
Small intestine	98.0	88.7*	83.9†	72.6†	90.1	121.0†	111.2*	86.4*	105.5
Large "	87.4	82.7*	90.0	86.5*	105.1	120.6*	116.0*	97.5	97.8
Thymus	101.8	86.4	42.2†	11.5†	22.2†	22.9†	23.5†	26.7†	43.4†
Depot fat‡	106.7	117.3	95.6	74.2	69.7	75.3	87.7	90.6	89.7
GI contents‡									
Chow	108.1	100.5	108.5	82.8	75.2	67.2	60.1	90.0	103.7
Synthetic	101.4	143.9	101.0	82.2	120.4	79.6	67.5	45.9	80.7

* Significant at 5% level ($.05 \geq P > .01$).
† " " 1% " ($P \leq .01$).
‡ See text for discussion of significance.

mals in a manner designed to prevent intergroup variations from introducing a consistent bias into any portion of the data. Both the normal control data and the procedures for determining tissue weights have been discussed previously(1). All tissue weights are expressed as a per cent of Wc, the body weight minus the weights of the gastro-intestinal contents and body fat(1), and the post-irradiation tissue weights are then expressed as a per cent of the corresponding control tissue weights. This method of expressing the data overcomes artifacts resulting from the large changes in gastrointestinal content weights observed in the post-irradiation period(2). The actual calculation can best be demonstrated by using an example. The average control weight of the heart (after the blood had been expressed from the chambers) was 0.332 ± .030% of Wc(1). The weight of the heart of rat #88 (body wt = 350.2 g pre x-ray and 316.0 g at dissection) at 2 days post x-ray was 1.024 g. The average control body fat and gastrointestinal contents weights were 15.8 and 19.7 g, respectively, for this experiment. Thus, for rat #88, the heart was

$$\frac{1.024 \times 100}{350.2 - 15.8 - 19.7} = 0.325\% \text{ of control Wc.}$$

This figure averaged with similar data from 6 other rats yielded a mean of 0.342% of control Wc, which, as seen in Table I, was 103.0% of the normal control figure and did not differ significantly from it. The data in Table I represent the averages of over 1600 tissue weights from 193 different rats.

Results. The details of the tissue weight loss at each period following total body irradiation are shown in Table I. Each post-irradiation tissue value represents the mean of 7 ± 2 independent dissections, while the control values are the mean of 16 ± 5 independent determinations(1). The corresponding total body weights, expressed as a % of the pre-irradiation value, are 98.9, 98.2, 98.6, 92.2, 88.2, 87.0, 87.9, 90.5, and 94.5% respectively for 3 hrs, 12 hrs, and 1, 2, 3, 4, 6, 8, and 10 days following x-irradiation.

The loss of body weight following total body x-irradiation is primarily associated with a loss of muscle mass. The skeletal muscle is the only major tissue in which there is a prompt, progressive, and large loss of weight. The losses in liver and body fat weight are small and delayed. This pattern of loss constitutes a major difference between the weight loss characteristic of radiation and that seen in starvation(2). The tissues to lose the greatest amount of weight, on a relative basis, are the spleen and thymus. The thymus, in fact, comes so close to disappearing at 1 and 2 days following irradiation that its exact dissection and weighing become matters of considerable uncertainty. The total initial weight of both the spleen and thymus is so small, however, that this change has little significance in terms of body weight loss. The changes in the weight of the skin, kidneys and the liver roughly parallel that observed for total body weight. Thus, at each period these tissues represent a nearly constant per cent of the body weight at that time. The gastrointestinal tract shows alternate periods of increase and decrease in weight. The periods of decreased weight correspond roughly with the early and late irradiation death periods.

There are significant increases in the weight of supportive tissue following total body x-irradiation. This is due to the increased hydration of the connective tissue, rather than to any change in the weight of the mineral mass. Complete dissections demonstrated that there was no significant alteration in the ash, dry, or fresh weights of the skeleton or teeth at any of these periods. From the potassium and desoxyribonucleic acid data of Fig. 1, there appears to be a rapid loss of about 30% of the active marrow following x-irradiation. The near equivalence of these two curves indicates that a major part of the skeletal potassium is associated with marrow rather than bone mineral. The other curves in Fig. 1 show the increase in iron porphyrin content and then fat, associated with this destruction of marrow.

The weight of body fat(1) decreases significantly after the 2nd or 3rd day following irradiation. However, most of the body tissues, when carefully dissected, show no significant decrease in their fat content at any time, and marrow (Fig. 1) shows an increase. Thus, this decrease is confined to and characteristic of gross depot fat. The figures in Table I for body fat and gastrointestinal contents represent the means of several experiments which differed considerably in absolute terms. The same relative changes were present and statistically significant in each case, however.

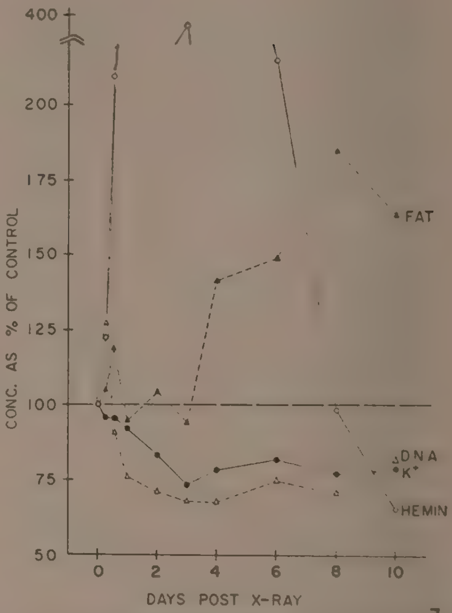

FIG. 1. Changes in composition of skeleton following total body x-irradiation.

Three-hour weight loss. During the process of irradiation the rats were confined, 4 at a time, to a Lucite box whose surface was perforated with 1 cm holes for ventilation. We routinely found a loss of 7.4 ± 4.7 g of body weight during the 0.5-.8 hr required for the irradiation. The question naturally arises as to whether or not this weight loss is attributable to the irradiation or is a result of the manner in which the animals are handled.

The pertinent data are shown in Fig. 2. Here the per cent weight loss observed under each of 4 different sets of experimental conditions is plotted against time in hours. There was no significant difference between the weight losses observed in the groups of rats that were: (a) confined to the Lucite box for 2 hrs without food and water, (b) left in their cages without food and water for 2 hrs, and (c) sham irradiated, *i.e.*, placed in a carrying cage, transported to the x-ray room, placed into the Lucite box for ¾ hr, and returned to the animal room. The weight loss of the irradiated group was significantly ($P = .05$)

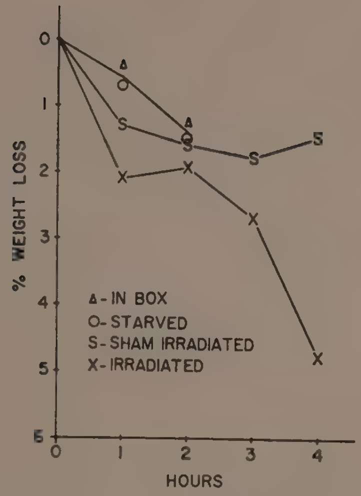

FIG. 2. Loss of body wt resulting from x-irradiation and from 3 control procedures.

greater than that observed for the sham-irradiated group during the first hour. During the next hour the animals were returned to their cages and they ingested some food and water. There was a slight weight gain, but this was promptly reversed, and after 3 hrs the weight of the irradiated group became progressively lower while the sham-irradiated animals recovered lost weight. As seen in Table I, much of the weight loss of the irradiated animal, even at this early period, can be explained in terms of a loss of muscle mass.

Summary. The weight changes observed in 15 tissues at 9 different periods following total body irradiation with an LD$_{50}$ dose (700 r) of x-ray have been described and statistically evaluated. The loss of muscle mass explains the major portion of the body weight loss which is seen in the rat during the first 4-6 days following irradiation. Though the percentage weight loss was greater in spleen, thymus and marrow, these tissues are too small to have an appreciable effect upon total body weight change.

The authors wish to thank Judith Poncelet and Ada B. Simon for their technical assistance, and Drs. K. W. Stenstrom and J. F. Marvin of the Department of Radiology for x-irradiating the rats used in this study. This work was carried out under Contract AT(11-1)-106 between the University of Minnesota and the Atomic Energy Commission.

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Vit. B$_{12}$ Distribution, Determined by Surface Body Counting Following Parenteral Administration of Co^{60} Vit. B$_{12}$.* (22188)

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In a preliminary report it was stated(1) that following intramuscular injection of 1 μg of Co^{60} vit. B$_{12}$ maximal radioactivity was detected over the liver, spleen and left kidney, in the order named. Activity persisted over the hepatic area during periods of observation in all cases, and ranged up to 315 days. More recently, Glass and his co-workers reported studies in subjects who received Co^{60} vit. B$_{12}$ parenterally(2). Their results showed essentially the same pattern of distribution as those previously described from our laboratory.

The present report is a detailed description of our findings on a large group of patients, and a comparison of these results with 6 persons receiving Co^{60} Cl$_2$.

Materials and methods. The first batch of Co^{60} vit. B$_{12}$ had a specific activity of 0.240

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* Co^{60} Vit. B$_{12}$ and Co^{60} Cl$_2$ were supplied by Merck and Co., Rahway, N. J.

TABLE I. Distribution of Radioactivity following Intramuscular Injection of 1 μg of Co^{60} Vit. B_{12} (Containing 0.045 μg Co).

				Radioactivity over liver			Radioactivity over spleen			Radioactivity over left kidney		
Age	Diagnosis	Observation, days	Disappearance from site of inj., hr	Max activity after inj., days	Duration of plateau, days	Total activity, days	Max activity after inj., days	Duration of plateau, days	Total activity, days	Max activity after inj., hr	Duration of plateau, days	Total activity, days
47	Normal	315	4	3	44	315	3	37	166	4	37	166
30	"	98	3	3	34	98	3	27	98	1	37	55
79	Arteriosclerosis	64	3	1	63	64	1	56	64	2	3	64
63	Compensated cardiac	145	3	1	144	145	3	82	122	1	26	89
58	*Idem*	131	3	3	101	131	1	54	131	4	34	55
47	Rheumatoid arthritis	155	3	2	72	155	4 hr	71	141	1	29	64
49	*Idem*	157	4	1	156	157	6 "	47	157	3	47	69
52	Cirrhosis of liver	61	3	2	27	61	1 "	52	61	1	40	61
64	*Idem*	182	3	4 (hr)	133	182	30 min.	29	182	30 min.	15	90
75	P. A. untreated	93	3	1	92	93	1	78	93	1	16	93
50	*Idem*	11	3	2	9	11	4 hr	11	11	4	5	11
61	Gaucher's disease	131	3	1	130	131	1	116	117	2	45	55
34	Lymphosarcoma	84	4	2	25	84	4 hr	41	84	4	8	27
45	Polycythemia	80	4	3	77	80	1	17	80	*	*	*

* Not counted.

TABLE II. Distribution of Radioactivity following Intramuscular Injection of 1 ml of Co^{60} Cl_2 (Containing 0.042 μg of Co).

				Radioactivity over liver			Radioactivity over spleen			Radioactivity over left kidney		
Age	Diagnosis	Observation, days	Disappearance from site of inj., days	Max activity after inj., hr	Duration of plateau, hr	Total activity, days	Max activity after inj., hr	Duration of plateau, hr	Total activity, days	Max activity after inj., hr	Duration of plateau, hr	Total activity, days
75	Hypertension	77	77	1	12	20	1	12	5	1	2	4
84	Arteriosclerosis	"	70	2	4	23	2	4	8	2	2	2
64	Congenital heart disease	"	77	2	24	55	1	24	20	8	12	2
64	Diabetes mellitus	"	70	1	6	13	1	24	13	1	6	1
77	P. A. untreated	42	13	2	4	7	2	3	3	2	4	4 hr
73	Cirrhosis of liver	"	42	2	12	20	2	4	7	4	4	24 "

$\mu c/\mu g$. At a later date this was increased to 0.760 $\mu c/\mu g$. Each dose was diluted with sterile distilled water so that 1 ml contained 1 μg of radiovitamin (0.045 μg Co/μg of vit. B_{12}). The Co^{60} Cl_2 contained 0.042 μg of Co^{60}/ml solution and had a specific activity of 1.86 $\mu c/\mu g$. All administered doses were 1 ml in volume and were injected into the right deltoid muscle, using a 1-inch 22 gauge needle. A well-shielded sodium iodine crystal scintillation counter having an aperture of 2 cm was placed over 1) site of injection (deltoid muscle), 2) precordium, 3) lower right chest (liver), 4) lower left chest (spleen), 5) left lumbar region (left kidney), 6) lower end of sacrum, 7) lateral aspect of thigh. Radioactivity was recorded for 5-minute periods over each site within ½, 1, 2, 3, 4, 8, 12, and 24 hours after injection. Thereafter, measurements were made at 2, 3, 5, and 7 day intervals for periods ranging from 11 to 315 days. Background counts were subtracted from observed values. No correction was made for Co^{60} decay, since even up to 315 days the correction for physical decay would amount to less than 15%. All but 4 patients were inmates of a chronic disease hospital and received the same diets unless precluded by specific therapy.

Results. Fourteen subjects received 1 μg of Co^{60} vit. B_{12} intramuscularly as described above. The results are tabulated in Table I. Since no significant level of radioactivity was observed over the precordium, sacrum and thighs, these sites were excluded from this table. Activity was always detectable over the liver, spleen and left kidney within 30 minutes after injection, so that the first column under each organ records time after injection when maximal number of counts was noted. In general the highest radioactivity was detectable over the liver, with less activity over the spleen and left kidney in the order named.

Six subjects received 1 ml of Co^{60} Cl_2 (containing 0.042 μg Co^{60}) intramuscularly. These data are shown in Table II. The pattern of absorption from the site of injection and distribution over the liver, spleen and kidney as measured by surface body counting are entirely different from those following Co^{60} vit. B_{12} administration.

From the above results it is apparent that with the wide variations in time required for achievement of maximal radioactivity after injection, duration of plateau and persistence of activity over the organs named, no clear-cut pattern was observed in healthy or diseased state. Age of subject did not appear to be a factor either. The results in 2 untreated cases of pernicious anemia, one of whom was in moderately severe relapse, were most surprising, since radioactivity was detectable over the liver for as long as individuals were studied.

Since previous animal studies have shown that following parenteral administration of Co^{60} vit. B_{12} to rats, radiovitamin is found in the liver, spleen and kidney after 3 to 4 days (3,4), it is probable that the radioactivity measured in the present studies represent the B_{12} complex with Co^{60} still attached. The results with Co^{60} Cl_2 help to confirm this conclusion.

Conclusions. 1. Following intramuscular administration of 1 μg of Co^{60} vit. B_{12} to normal persons and patients with various medical disorders, radioactivity disappeared from site of injection in 3 to 4 hours. 2. Maximal radioactivity was detected over the liver, spleen and left kidney regardless of clinical condition or age of subject. The wide variations preclude any typical pattern for normal or diseased states. Previous animal studies with Co^{60} vit. B_{12} and present investigations with Co^{60} Cl_2 suggest that the measured radioactivity represents vit. B_{12} complex in organs named.

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Poliomyelitis Antibodies in Inhabitants of the Society Islands, French Oceania. (22189)

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In 1950, a study had been planned of the distribution of poliomyelitis serum-antibodies among inhabitants of the Society Islands, French Oceania. Unfortunately, the first serum samples obtained were lost in transit to the United States. Then, in March 1951, an unexpected outbreak of the disease occurred in Tahiti, largest of the islands. Collections of new specimens from this and other islands, therefore, had to be conducted during and after the epidemic period. This was the first poliomyelitis epidemic reported from the region, although several sporadic cases of the disease were known to have occurred prior to 1951(1,2).

The accompanying map (Fig. 1) shows the relative position of the Society Islands and distances between them. There is daily boat service between Tahiti and Moorea, while only weekly service operates between Tahiti, Huahine, Raiatea and Bora-Bora. Irregular service takes place in smaller boats by special arrangement between Bora-Bora and Maupiti—perhaps every 2 weeks or only once a month. Maiao is not in direct line of travel between Tahiti and the larger islands and, because of its small population and inadequate pass, boats do not call there more than 3 or 4 times a year, and always by special arrangement from Tahiti. Tahiti, where the epidemic began, reported 109 cases of the disease, and the other Society Islands, only 2 cases: one from Huahine and another from Raiatea. No paralytic cases were observed in Maiao, Maupiti or Moorea. The high paralytic rate of 360 per 100000 (1,2) was recorded during this epidemic.

The object of this communication is to present results of attempts to isolate virus from stool specimens collected from several frank cases of the disease and to measure complement-fixing and neutralizing antibodies in serum specimens from Tahitian patients as well as from the general populations of Maiao, Maupiti, Moorea and Huahine.

Materials and methods. Stool specimens

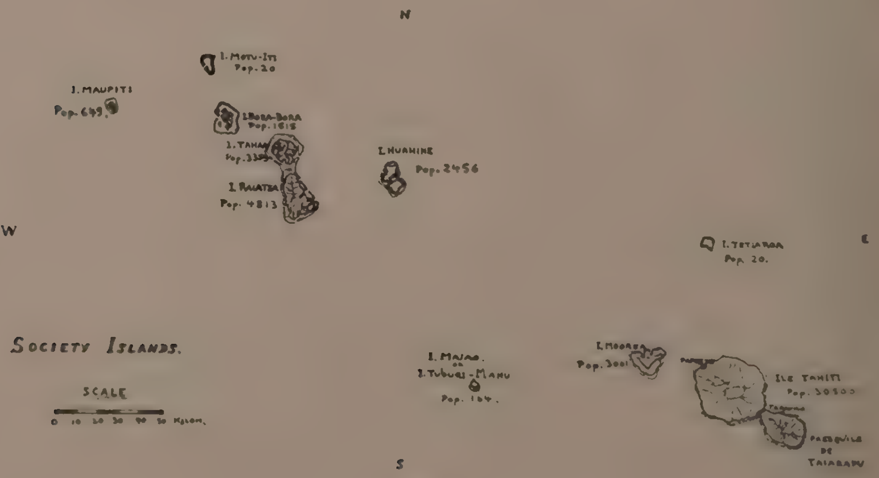

FIG. 1.

TABLE I. Schedule of Collection and Testing of Serum Specimens from the Society Islands.

	Total No. of sera collected		No. of specimens tested			
			Polio patients		Others	
Island	Polio patients	Others	C.F.	S.N.	C.F.	S.N.
Tahiti	10	31	8	6	31	6
Maiao		21			20	12
Maupiti		71			46	12
Moorea		76			55	12
Huahine		72			65	15
Totals	10	271	8	6	217	57

were secured from 7 sick patients in Tahiti and all were processed for virus isolation.* A total of 281 serum samples were collected from 5 islands (Tahiti, Maiao, Maupiti, Moorea, and Huahine). Because of breakage upon arrival at the laboratory, only 217 of these were tested for Type 2 antibodies by the C.F. method. Of these, a great number were found anticomplementary, and data reported below pertains to 131 individuals. In addition, 57 specimens were tested for all 3 types of antibodies by the serum-neutralization method (Table I). *Complement-fixation.*† The technic used was essentially the Kolmer-Boerner test(4) which utilizes overnight icebox incubation. Inactivated serum specimens were diluted in normal saline in serial 2-fold dilutions from 1:2 to 1:16. Duplicates of the 1:2 and 1:4 dilutions were also made. Addition of 2 units of Type 2 antigen to the complete line of tubes and of a corresponding volume of saline to the duplicates followed. After distribution of 2 full units of complement, incubation was carried out at 4°C for almost 18 hours. Two per cent sheep cells and 2 units of hemolysin were then added, and the tubes were read after further incubation for 15 minutes at 37°C. The antigen used was derived from acetone-extracted material from suckling hamster brains infected with the MEF_1 strain of Type 2 virus. *Serum Neutralization.*† This was done by mixing equal amounts of inactivated, undiluted serum with each of 3 tissue culture suspensions containing about 200 tissue culture doses of Type 1, 2, or 3 virus, giving final concentrations of 1:2 serum and 100 infective doses of virus. In a few cases, where the amount of serum available was limited, initial 1:2 dilutions were used in the mixtures. Adequate normal serum control mixtures were also prepared. All mixtures were incubated in water bath at 37°C for one hour before inoculation into monolayer, monkey kidney culture tubes—3 or 4 per mixture—in medium 199(5). Virus titrations were performed simultaneously. The tubes were observed for cytopathic action for 4 days, at which time the cells of control tubes were completely destroyed.

Results. Of the 7 stool specimens tested, only one yielded poliomyelitis virus of Type 1. This was from the same patient also found to be positive by Dr. Horstmann(3) with whom each of the specimens had been divided.

Results of neutralization tests against all 3 types of poliomyelitis virus on sera collected from individuals on the islands of Maiao, Maupiti and Huahine; and on sera from paralytic cases in Tahiti, obtained from one to 5 weeks after onset of illness, are shown in Fig. 2. It must be remembered that Maiao (column 1) has the least communication with Tahiti and the other Society Islands, whereas Maupiti (column 2) has slightly more, and Moorea and Huahine (columns 3 and 4) are visited by ships at daily or weekly intervals.

Since Maiao remained isolated early in the epidemic, it may be assumed that the neutralization pattern for sera from this island is representative of that which prevailed in all of the Society Islands prior to the 1951 epidemic. Types 1 and 2 viruses were neutralized by less than 30% of the serum samples from Maiao while Type 3 virus was neutralized by 75% of the samples. In addition, analysis of the Maiao results by age groups reveals a total absence of Type 1 neutralizing antibodies in individuals below 25 years of age.

Tahiti presented a different picture, with 93% of the sera from recognized cases of poliomyelitis collected during the epidemic,

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* Courtesy of Dr. Charles F. Pait and Dr. Penti Kokko, Virus Laboratory, Los Angeles County Hospital.

† These tests were performed by the Research Division, Lederle Laboratories, Pearl River, N. Y.

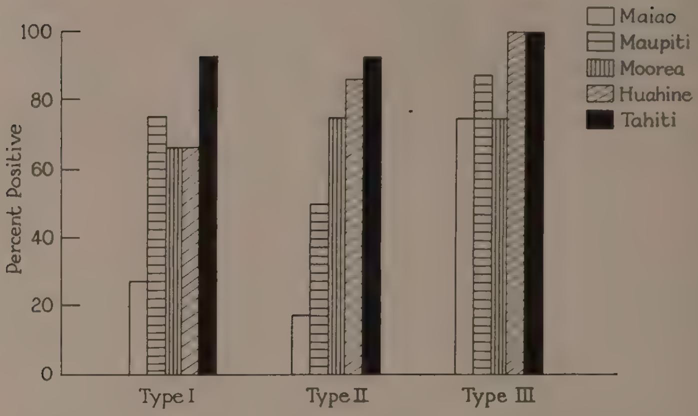

FIG. 2. Types I, II and III neutralizing antibodies in Society Islands.

neutralizing Types 1 and 2 viruses, and 100% neutralizing Type 3 virus. As might have been expected, specimens from other islands showed patterns intermediate between those found for Maiao and Tahiti.

The isolation of Type 1 virus from the stool specimen of one of the patients with paralytic poliomyelitis suggests that this virus was active at the time and that an increase of antibodies to Type 1 virus was to be expected during or after the epidemic. That this occurred is indicated by the fact that 93% of the blood samples collected from post-paralytic cases showed neutralizing antibodies to Type 1 virus, the highest rate in any group tested. It is of interest that the rate of Type 2 neutralizing antibodies was also high in the same group of specimens—93%. In addition, when these sera were tested for complement-fixing antibodies, 75% were found positive for Type 2 virus.

Distribution of neutralizing antibodies by age groups for the 3 types of virus, and of complement-fixing antibodies for Type 2 among sera tested, is depicted in Fig. 3. Specimens from active cases of poliomyelitis infection were excluded from this Figure. It will be observed that, except for the youngest age group, 0-4 years of age, the percentage of Type 3 neutralizing antibodies was higher than of Type 1 or 2; and that the patterns of neutralizing antibodies for Types 1 and 2 were similar to each other.

It is of interest that, shortly after the epidemic of poliomyelitis in Tahiti, Type 2 complement-fixing antibodies were present in normal sera collected from all islands surveyed. Since Casals and others(6) have shown that an increase of complement-fixing antibodies to Type 2 virus may be stimulated by the heterologous Type 1, it is possible that the presence of complement-fixing antibodies to Type 2 virus in the Society Islands at this time was stimulated by Type 1 virus which was active in these islands.

Summary and conclusions. Early in 1951, an epidemic of poliomyelitis occurred in Tahiti during which Type 1 virus was recovered from feces of a paralytic case. Tests on 131 serum samples collected from inhabitants of Tahiti and others of the Society Islands, during and after the outbreak, yielded the following results: 1. A higher percentage of Type 3 than of Types 1 and 2 neutralizing antibodies was found in sera from persons living in the outer Society Islands. On the other hand, higher percentages of neutralizing antibodies against Type 1 and 2 viruses were present in sera of residents of Tahiti during and after paralytic attacks of poliomyelitis than in normal serums from the outer islands. 2. After the epidemic, complement-fixing antibodies for Type 2 virus were present in sera of individuals from the outer islands, and in even higher percentages in sera of post-paralytic patients in Tahiti. Since complement-

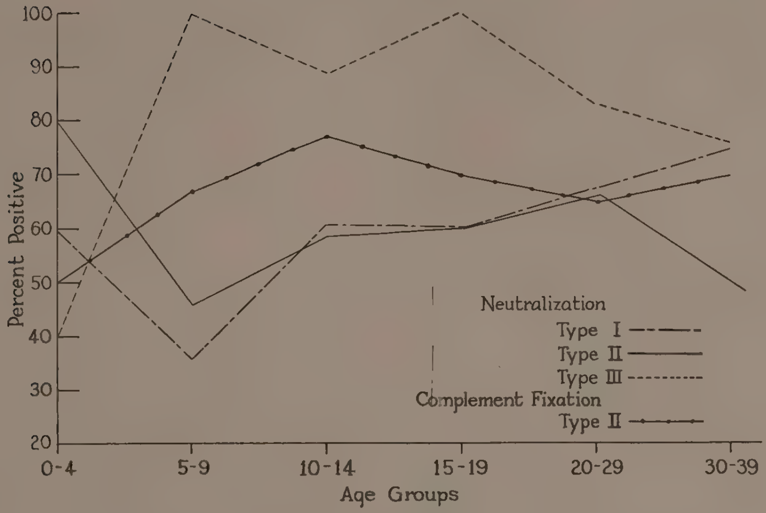

FIG. 3. Neutralizing antibodies by age groups for Types I, II and III and complement fixing antibodies for Type II virus.

fixing antibodies to Type 2 virus may indicate serologic response to the heterologous Type 1 virus, widespread dissemination of Type 1 virus probably occurred at this time. 3. These observations suggest that immunity to poliomyelitis in the Society Islands prior to the epidemic of 1951 was greater to Type 3 virus, and that the outbreak resulted from recent introduction of Type 1 virus, a new type for the area. It is possible that Type 2 virus was present coincidentally.

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Modification of Radiation Injury in the Rabbit. (22190)

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Heterologous tissue transplants or cell suspensions of hematopoietic origin have been reported by Lorenz and his associates(1,2) and Jacobson *et al.*(3) to have a significant effect on survival and hematopoietic regeneration of irradiated rodents. Jacobson first described evidence that suggested the effectiveness of mouse spleen transplants for hastening recovery of the blood-forming tissue of irradiated rabbits(4). Congdon and Lorenz (5) reported that rat bone marrow and rat bone had a significant effect on survival of irradiated mice and that guinea pig marrow likewise afforded a significant increase in survival of irradiated mice. Jacobson and coworkers(3) and more recently Cole *et al.*(6)

corroborated the work of Congdon and Lorenz on effectiveness of rat marrow on survival of irradiated mice, but Loutit(7) has been unable to confirm these positive results with heterospecific material.

In earlier experiments, Jacobson did not observe the effect of mouse hematopoietic tissue transplants on survival of irradiated rabbits(4). Hence, the investigation described in this paper was undertaken to determine whether hematopoietic cells from mice enhance survival of X-irradiated rabbits.

Materials and methods. Swift's snuffle-free, young adult rabbits of both sexes, weighing 1.5 to 2.5 kg, were used as recipient animals. The cells used for postirradiation injection were obtained from tissues of the CF No. 1 mouse embryo or from baby mouse spleens or livers. The donor mice were killed by cervical fracture. Embryo tissue was obtained by making a median incision in the abdominal cavity of the pregnant mouse and removing and plunging the entire embryonic sac into cold Locke's solution. The liver was immediately excised from the embryo and suspended in Locke's solution. A homogeneous cell suspension was produced by drawing the combination into a 2-cc hypodermic syringe and expelling it repeatedly. Before cell counts were made, the suspension was drawn into a syringe and expelled through a 26-gauge needle. No appreciable cell clumping was found. The number of nucleated cells per cu mm was determined by making a dilution with 2% acetic acid in a white blood-counting pipette and counting the cells on a standard hemacytometer. Cell suspensions were diluted with Locke's solution so that each 10 cc contained the required number of cells for each rabbit. All injections were given within a few minutes after final dilutions had been made and were administered intravenously into the marginal ear vein of rabbit with a 24-gauge needle within a few hours (0 to 4) after X irradiation.

Cells from spleen, liver, and other tissues of the mouse were obtained in similar fashion. Cells obtained from baby mouse liver or spleen were not contaminated with other mouse tissue cells.

TABLE I. Outline of Experiment to Study Hematologic Changes in Irradiated Rabbits Treated with Cell Suspensions of Mouse Tissue.

Mouse tissue	Total No. cells ($\times 10^6$)	Rabbits (No.)	X-ray exposure (r)	30-day survivors (No.)
None	None	3	None	3
1-day liver	250	4	900	2
1- to 4-day spleen	209	2	"	1
16-day embryo liver	240–255	6	"	5
None	None	4	"	1

Rabbits were exposed to 900 r total-body X radiation* before administration of the cell suspensions. Irradiated rabbits that were injected with cell suspensions from nonhematopoietic tissue and nonirradiated rabbits that were untreated served as controls. The experiment to study hematologic recovery of irradiated rabbits following injection of cell suspensions obtained from various tissues of the mouse is outlined in Table I. Table II gives a detailed outline of experiments devoted to a study of survival. As indicated in Table I hematologic studies were made on a small number of animals. Only one control irradiated animal survived beyond the 14th day. Leucocyte, hematocrit. and reticulocyte values were determined for irradiated rabbits that had been injected with mouse embryo liver or with baby liver or spleen; for irradiated, untreated rabbits; and for rabbits that were normal, non-irradiated, untreated controls (Table I). Standard hematologic technics were employed. X-rays were administered as a horizontal beam to 2 animals at a time. Measurements were made in air with a Victoreen condenser r-meter equipped with a 100-r chamber at the position occupied by center of the 2 cages. At the midpoint of total exposure, the animals were reversed from top to bottom and from side to side. X-rays were generated by a 250-kvp Maxitron machine operating at 30 ma. A 1.0-mm Al and 0.5-mm Cu filter were used. The half-value layer in copper of the filtered beam was 1.43 mm. Exposure rate averaged 44 r per minute at a

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* The 30-day X-ray LD50 for these animals in this laboratory is about 800 r.

TABLE II. Effect of Cell Suspensions of Mouse Tissue on Survival of Rabbits Exposed to 900 r Total-Body X Radiation.

Mouse tissue	Total No. cells (× 10⁶)	Rabbits (No.)	Survival 28-day No.	%	120- to 150-day No.	%
Embryo liver	200 to 250	19	11	57.8	10	52.6
1- to 2-day liver	"	10	6	60	6	60
1- to 4-day spleen	25 to 30	8	6	75	6	75
Heart, kidney, lung, placenta	32	9	1	11	1	11
None	X-ray only	50	5	10	4	10

distance of 70 cm.

Results. Survival. As indicated in Table II, cells from the mouse embryo or from baby mouse spleen or liver had an appreciable effect on survival of rabbits that had been exposed previously to 900 r total-body X radiation. Of 8 irradiated rabbits that received from 25 to 30 x 10^6 cells from the spleen of 1- to 4-day-old mice, 6 have survived a period of 150 days, whereas only 1 of 9 has survived 120 days that was injected with cells from nonhematopoietic tissue. All nonsurvivors died within the first 26 days of observation period, and no deaths have occurred since that time. Rabbits that received from 200 to 250 x 10^6 cells from the liver of the mouse embryo or from liver of the 1- to 2-day-old mouse had similar survival. Of 29 thus treated, 17 survived beyond 26 days, and only 1 death has occurred (on day 90) during remaining 120-day period of observation. Of 50 untreated irradiated controls, only 4 survived (Table II).

Hematologic studies. Figs. 1 and 2 indicate that injection of 200 to 240 x 10^6 cells from the mouse embryo or baby liver or spleen had no appreciable effect on recovery of leucocyte and hematocrit values of the peripheral blood of the irradiated rabbit. The normal reticulocyte value of the irradiated cell-injected group at 10 days as compared with controls (Fig. 3) may be significant but should not be considered as evidence of an earlier recovery in these animals unless confirmed by histopathologic findings or a further hematologic study in which values are obtained at additional periods.

Discussion. The data from this investigation indicate that cells from the embryonic mouse liver or from baby mouse liver or

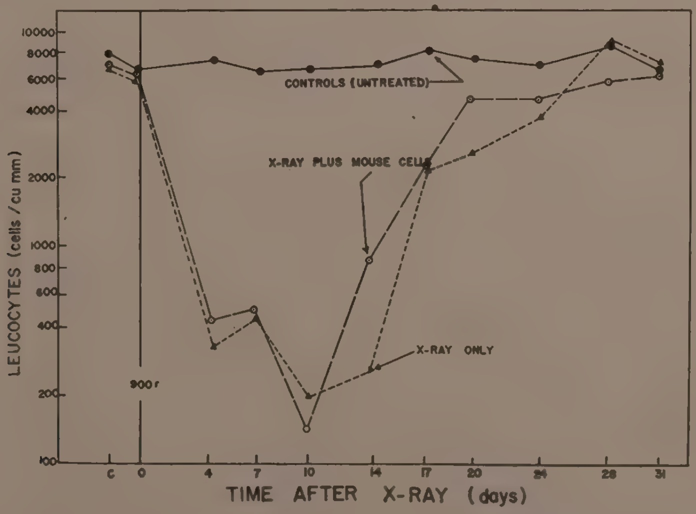

FIG. 1. Effect of cell injections on recovery of irradiated rabbits.

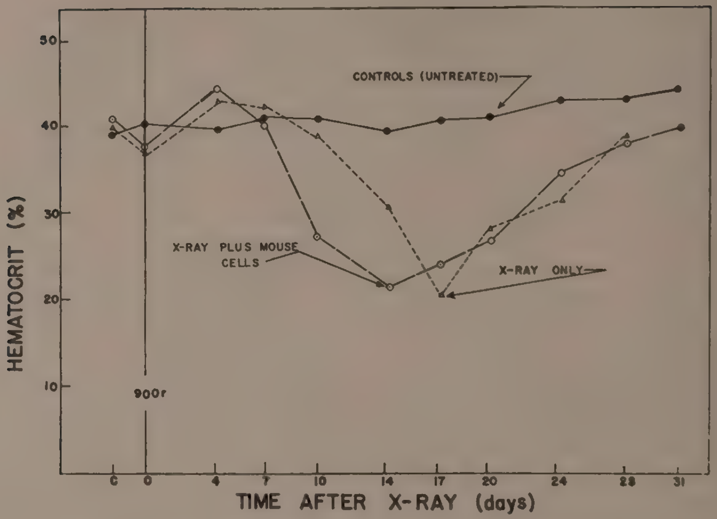

FIG. 2. Effect of cell injections on recovery of irradiated rabbits.

spleen are capable of enhancing the survival of rabbits exposed to 900 r total-body X radiation. These findings supplement those of Lorenz(1,2), Jacobson and his associates (3,4), and Cole *et al.*(6) which show the efficacy of heterologous tissue (rat to mouse, guinea pig to mouse) in prolonging the survival of irradiated mice. Loutit(7), on the contrary, has not been able to increase the survival of irradiated mice by treating them with heterospecific material. In point of fact, his argument against the benefits of homologous suspensions is based upon the number of late deaths that he observed in irradiated mice that had been treated with cell suspensions made from animals of the same species but of a different strain. It is thus important to emphasize that, in our study, late deaths did not

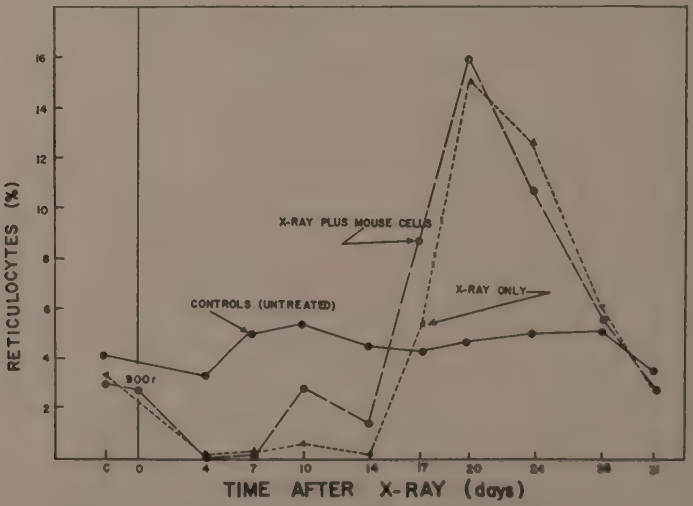

FIG. 3. Effect of cell injections on recovery of irradiated rabbits.

occur in the surviving group of irradiated rabbits that had been treated with mouse tissue.

Previous studies in this laboratory gave evidence that spleen-shielding had only a slight effect on survival of X-irradiated rabbits(8), whereas there was an appreciable effect on survival of mice(4). The possibility that hematopoietic tissue from the mouse is a more potent source of the recovery factor (s) involved in recovery from radiation injury than hematopoietic tissue from the rabbit will require further exploration. Preliminary data in our laboratory suggest that mouse cells from embryonic liver as used in this study are as effective in enhancing survival of irradiated rabbits as are rabbit cells obtained from the liver of 1-day-old rabbits.

The fact that hematologic recovery in the rabbit exposed to 900 r and then treated with cells from the mouse was not appreciably different from that of control rabbits that survived this exposure would seem to indicate that increased survival is not necessarily dependent upon enhanced hematopoietic regeneration. Until histopathologic studies are completed it is perhaps not justified to make such a statement. Evidence of recovery may be present in the bone marrow and elsewhere under conditions of this experiment which are not manifest in peripheral blood.

In some respects, our findings are similar to those from the spleen-shielding splenectomy experiments that have been reported previously(4). Survival of spleen-shielded mice (1025 r) that were splenectomized 1 hour after irradiation spleen-shielding procedure was the same (75%) as that of mice treated similarly, with the exception of splenectomy. Hematopoietic regeneration began by 3 days and was complete in the nonsplectomized spleen-shielded mice in 7 to 9 days, while in mice splenectomized 5 minutes after the spleen-shielding-irradiation procedure, regeneration began at 9 days and was complete at 15 days or longer. These observations suggested that increased survival was not necessarily dependent on early regeneration of the blood-forming tissue. Our finding also suggests that early hematologic recovery is not necessary for increased survival.

Conclusions. Postirradiation injection of mouse embryo cells or baby mouse liver or spleen cells enhances significantly the survival of rabbits exposed previously to 900 r total-body X radiation without producing an appreciable effect upon hematologic recovery as observed in the peripheral blood.

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Metabolism of $NaHC^{14}O_3$: Effects of Anesthesia and Route of Dosage.*
(22191)

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A number of workers(1,2) have studied health physics aspects of $NaHC^{14}O_3$ metabolism and others(3,4) have shown bicarbonate carbon to be fixed in a variety of metabolites. We were unable to find adequate information in the literature on early times of the time course of $C^{14}O_2$ excretion or of effects of anesthesia upon this excretion. The effect of a further variable, route of dosage, also does not appear to have been reported. For adequate interpretation of the fate of labeled metabolites that are oxidized, it is necessary to know the fate of $C^{14}O_2$ so produced. In a following study important use is made of the time course of $C^{14}O_2$ excretion following acetate dosage.

Methods. Rats were given tracer $NaHC^{14}O_3$ under a variety of conditions and respired $C^{14}O_2$ and $C^{12}O_2$ were measured. Following multiple dosage of the $HC^{14}O_3^-$ the rats were sacrificed and tissue lipides analyzed for C^{14} activity. Sprague-Dawley, adult, male, 200-g white rats trained to feeding as before(5) were used exactly one hour postprandially. Following injection, the rats were placed in the metabolism chamber of the apparatus(6) and respiratory $C^{14}O_2$ activity data collected from the decimal scaler component. Occasional one-minute CO_2 samples were collected. The total CO_2 and $C^{14}O_2$ exhaled was collected and the amount and activity of the CO_2 was determined. Animals anesthetized were given 3.5 mg/100 g body weight of Nembutal. The anesthesia was given 45 minutes after start of the one-hour eating period, which was thus 15 minutes before the tracer bicarbonate was given. Radiobicarbonate was prepared by acidification of Oak Ridge $BaCO_3$ and absorption of the released CO_2 in NaOH. The pH of the solution was 8.2 and contained 0.38 mg $NaHCO_3$/ml with an activity of 10.84 μc/ml; 0.4 ml was the usual dose. Aliquots of the standard $NaHCO_3$ solution and respiratory CO_2 samples collected in alkali were directly plated(7) and counted under a thin end window tube as before(8). Because of low activities found in tissue samples, the fatty acids were directly plated, the cholesterol was plated as the digitonide and liver glycogen was also directly plated. Counting rates were corrected to "infinite thickness" by the application of appropriate factors previously determined in this laboratory. Specific activity figures listed below are on the order of one-tenth of the specific activities obtainable at "infinite thinness." A total of 9 experiments was conducted on 2 rats. Since each animal was subjected to the 3 conditions studied, "internal control" of experimental conditions was maintained. Intravenous injections were made in the saphenous vein at the junction of saphenous and lateral plantar veins. This route of injection permits a rapid yet quantitative administration of label. Following intravenous injection of HCO_3^- the animal was put into the metabolism system with dispatch because of the rapid rate of $C^{14}O_2$ excretion. The greater part of the bicarbonate dose having been exhaled in 3 hours allowed us to reuse the rats 24 to 48 hours after previous dosage. Forty-eight hours following the second dose of bicar-

TABLE I. Dosage Schedule for the Two Rats Receiving Tracer Bicarbonate.

Exp.	Animal No.	Dose, ml	Route	Anesthesia
1	V5-1	.4	I.P.	—
2	2	"	"	+
3	3	"	"	—
4	4	"	I.V.	+
5	V6-1	.8	I.P.	—
6	2	.4	"	+
7	3	"	"	—
8	4	"	"	—
9	5	"	I.V.	+

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* This investigation was supported largely by contract No. AT(45-1)-225 of the U. S. Atomic Energy Commission.

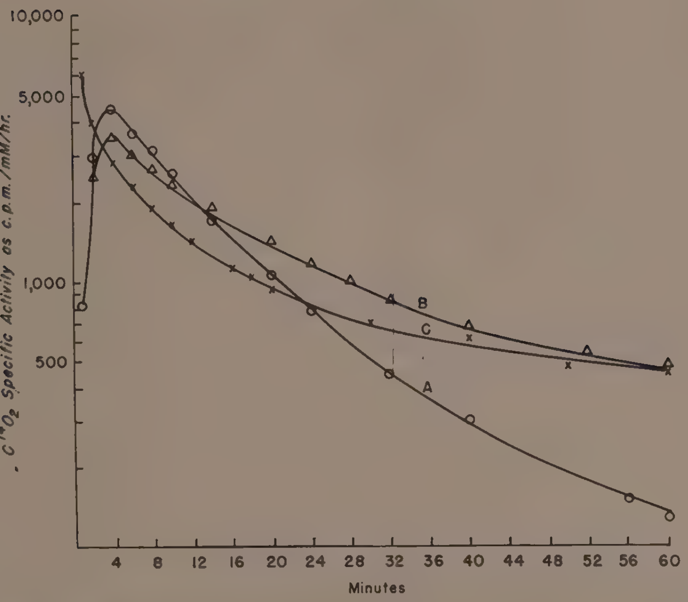

FIG. 1. Respiratory $C^{14}O_2$ activities following $NaHC^{14}O_3$ dosage. Curve A, avg of 4 experiments with 2 rats, 0.4 ml $NaHCO_3$ intraperitoneally. Curve B, as with the experiments of A but with rats anesthetized with Nembutal. Curve C, avg of 2 experiments in which anesthetized animals were given the tracer $NaHC^{14}O_3$ intravenously.

bonate the animals were placed in the metabolism chamber and respiratory $C^{14}O_2$ activity monitored. No activity above background was detected.

Results. Table I presents the details of the nine experiments from which time-course CO_2 data were obtained. Exp. 1 and 3 represent duplicate intraperitoneal, nonanesthetized runs on the first animal and Exp. 7 and 8 similar duplicate runs on second animal, giving a total of 4 trials of these conditions. Exp. 2 and 4 tested the effects of Nembutal upon $C^{14}O_2$ excretion after intraperitoneal dosage. Runs 4 and 9 tested the combined effects of Nembutal and intravenous dosage. The effect of dosage size was tested in Exp. 1 and 5.

The c.p.m. obtained from the decimal scaler(6), were converted to specific activities by dividing the actual count by the mM CO_2 produced per hour. This latter figure was obtained from the amount of CO_2 collected during the entire experiment. In each experiment 2, one-minute samples of CO_2 were collected and analyzed separately. The amount of CO_2 produced per minute measured by these samples agreed, within the usual range of such values, with the average CO_2 figures obtained from the total CO_2 collection.

The specific activity values used as the ordinate of Fig. 1 represent c.p.m. per mM CO_2 produced per hour, the counts used, being those recorded by the gas flow counting of the system described. These counting rates can be converted to equivalent infinitely thick $BaCO_3$ counting rates by multiplying by a factor of 5.3.

In Fig. 1 are presented the 3 curves that represent average results obtained from experiments detailed in Table I. Curve A represents the time-course of respired $C^{14}O_2$ following intraperitoneal dosage without anesthesia and curve B the effect of Nembutal anesthesia upon the $C^{14}O_2$ excretion. Following the intraperitoneal dosage both with and without anesthesia, $C^{14}O_2$ specific activities quickly increased and reached a maximum in about 6 minutes, demonstrating the rapidity of peritoneal absorption processes. Attainment of similar maxima at similar times suggests that the intraperitoneally administered Nembutal did not greatly affect HCO_3^- absorption from the peritoneal space. The $C^{14}O_2$ specific activities decreased following the maxima but plots of log s.a., $\log\frac{1}{\text{s.a.}}$, or $\log\frac{1}{\text{s.a.}^2}$ fail to reveal a straight line function for this decay, indicating the complex nature of the simultaneous distribution and excretion of the labeled bicarbonate. In the period immediately following the maxima, 7 minutes were required to reduce by ½ the s.a. of the intraperitoneal animals not anesthetized while 11 minutes were required for the anesthetized animals to reduce their $C^{14}O_2$ specific activities to one-half. Also, the anesthetized animals excreted only some 85% as much of the dose as did the nonanesthetized animals in the first hour. The decreased "turnover" of the HCO_3^- in the anesthetized rats is consistent with the slowing of respiration associated with Nembutal anesthesia. Upon awakening, in the 40- to 60-minute period, the rat treated with Nembutal struggles briefly and hyperventilates, which results in a sharp upward deflection in CO_2 specific activities. Following this, the respiratory rate of the rat slows and approximates that of the non-Nembutalized animal, for the slopes of the CO_2 specific activity curves become similar.

Curve C of Fig. 1 presents the average of the $C^{14}O_2$ time-course curves of the 2 experiments in which anesthetized animals were given bicarbonate intravenously. $C^{14}O_2$ appears in the respired air with such rapidity that it is difficult to make an accurate reading of the scaler at one minute. At this early time the respiratory CO_2 specific activity was found to be maximal, as might be expected from the probable maximum s.a. of blood HCO_3^-. The shape of the time-course curve plotted as C is similar in shape to curve B, confirming the effect of Nembutal anesthesia upon CO_2 turnover as seen in the intraperitoneally injected preparations.

Following the collection of CO_2 data in 4 experiments on animal V5 and in 5 experiments on animal V6, the rats were fractionated as previously described(5) and total saponifiable and nonsaponifiable lipide fractions isolated from liver, gut, carcass and skin. Liver glycogen was isolated from V5 and directly plated and counted as were the lipide fractions. The C^{14} activity of the lipides was extremely low and the data is thus not presented in detail. In the cholesterol (nonsaponifiable) fractions, the maximum per cent incorporation found was only 0.004. The maximum incorporation found for fatty acids (saponifiable fraction) was 0.05%. Compared to equivalent dosage with acetate-1-C^{14} in one hour postabsorptive animals, the cholesterol and fatty acid incorporation was less than 0.005 of that usually found. It is clear that the $C^{14}O_2$ released from tracer dosage, even from rapidly metabolized labeled substrates such as acetate, does not to a significant degree(4) label body fatty acids or cholesterol.

The liver glycogen of V5 after the 4 doses of tracer bicarbonate contained about 0.01% of the label. This figure has little absolute meaning because of the multiple nature of the dosage and the period of time elapsed during the 4 experiments.

Summary. The time-course of respiratory $C^{14}O_2$ has been followed after intraperitoneal and intravenous dosage of $NaHC^{14}O_3$ into anesthetized and non-anesthetized rats. Nembutal has been shown to affect the turnover of injected bicarbonate through its depression of respiration. The anesthetic did not slow peritoneal absorption. The multiple bicarbonate dosage used did not lead to appreciable labeling in tissue lipids, and the contribution of $C^{14}O_2$ to secondary labeling reactions can under these conditions be ignored. Liver glycogen "fixed" a small but measurable amount of the C^{14} label.

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Hyperglycemia and Increased Liver Glycogen in Rats After X-Irradiation.[*][†] (22192)

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It is well established that whole-body x-irradiation of rats results in an increase in level of liver glycogen(1-4). On the other hand it has been reported that liver glycogen of rabbits is markedly reduced following x-irradiation(5). Although x-irradiation raises liver glycogen level of rats and decreases that of rabbits, blood sugar levels in both animals are increased by exposure to x-irradiation. Kohn(6) found that in rats irradiated with 600-900 r, plasma glucose level rises within 2 to 3 days and McKee(4) has found, on the first day after irradiation, an increase in blood sugar levels of rats exposed to 1500 r. Steadman and Grimaldi(7) observed a hyperglycemia in rabbits following x-irradiation. This hyperglycemia increased with increasing dose and at every dose (500 to 2000 r) reached a maximum 4 hours after irradiation, thereafter declining to normal levels within 24 hours. Hyperglycemia was completely prevented by insulin. Therefore, these authors postulated that irradiation causes a mobilization of liver glycogen into glucose and that the normal supply of insulin in the rabbit is not sufficient to prevent hyperglycemia. Since irradiation increases liver glycogen levels in the rat, the question arises as to the reason for hyperglycemia. Before an attempt was made to answer this question, it appeared that a detailed study of the relationship of liver glycogen and blood sugar levels to dose and time after irradiation would be of value. Data on these points are presented in this paper.

Methods. Animals. Sprague-Dawley female rats about 10 weeks of age were used. In each experiment, animals of uniform weight were divided into groups of either 4 or 5 rats each. *Radiation technics.* Radiation source was Westinghouse X-ray machine of 250 KVP and 15 ma, with filter of 0.5 mm copper and 1 mm aluminum. All doses were delivered at 24.5 per minute at a target-to-skin distance of 40 inches. During irradiation rats were restrained in acetate-plastic boxes and slowly rotated under the beam to insure uniform exposure(8). Non-irradiated, control animals were caged in the same manner for the same period of time. *Experimental design.* In Exp. 1 only liver glycogen was determined. In Exp. 2 both liver glycogen and blood sugar levels were determined, whereas in Exp. 3 only blood sugar levels were measured. Rats subjected to whole-body x-irradiation were fasted for 24 hours, exposed to single x-ray dose and the fast continued. Doses administered were 500,

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[†] The opinions or assertions contained herein are private ones of the authors and are not to be construed as official, or reflecting the views of the Navy Department.

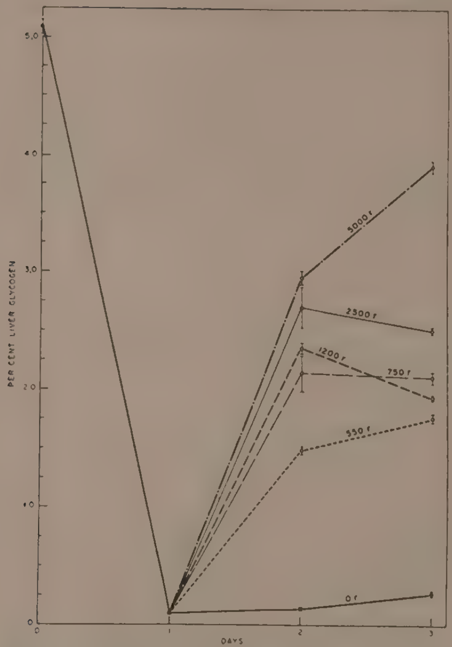

FIG. 1 (Exp. 1). Liver glycogen levels as a function of time in fasted, x-irradiated and fasted, normal rats. Fast was begun on day 0, and rats were exposed to x-radiations on day 1. There were 5 rats in each group of control animals and 4 rats in each group of fasted, x-irradiated animals. Means and stand. errors of means are plotted. Glycogen was determined by method of Good *et al.*(9).

750, 1200, 2500, and 5000 r. Eight rats were used at each dose level. Four rats from each group were sacrificed 24 and 48 hours after x-irradiation. Non-irradiated control animals were sacrificed in groups of 5 after 0, 24, 48, and 72 hours of fasting. *Analyses.* Rats were anesthetized with pentobarbital. Chest cavity was cut open and heart blood was withdrawn into a heparinized syringe. When liver glycogen was determined the liver was removed, blotted free of adhering blood and weighed. A liver sample of appropriate size was obtained, weighed and immediately analyzed for glycogen. In Exp. 1 glycogen was determined by the method of Good *et al.*(9). In subsequent experiments liver glycogen was determined by the method of Kemp(10) and blood sugar level by the method of Mendel *et al.* (11). The results are presented in Fig. 1 to 3.

Results. Liver glycogen levels. It is evident from Fig. 1 and 2 that fasting produces, within the first 24 hours, a sharp drop in percentage of liver glycogen. This drop is followed by a slight increase in liver glycogen during the next 48 hours. Whole-body x-irradiation, on the other hand, produces, in the fasting rat, a sharp rise in liver glycogen within 24 hours after time of exposure. The percentage of glycogen found in livers of irradiated rats increases with increasing dose. In each experiment the glycogen content of livers of rats exposed to 5000 r was considerably greater 48 hours after time of irradiation than at 24 hours. On the other hand, the percentage of glycogen found in livers of rats exposed to the other dose levels was about the same at both 24 and 48 hours after irradiation. At no time during the course of any experiment was the percentage of glycogen in the liver of the fasting, irradiated rat equal

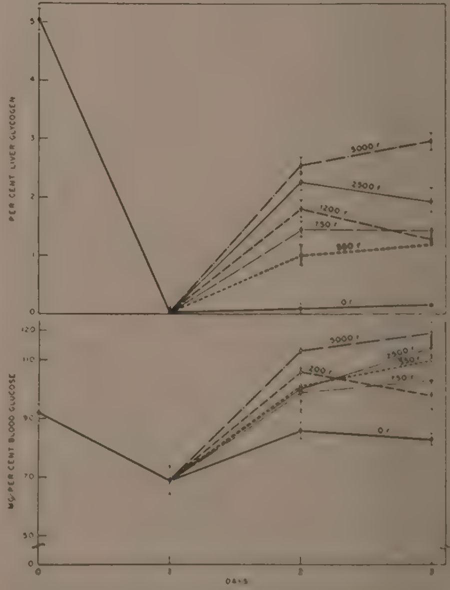

FIG. 2 (Exp. 2). Blood glucose and liver glycogen levels as a function of time in fasted, x-irradiated rats and normal control rats. Fast was begun on day 0 and rats were exposed to x-radiations on day 1. Five rats in each group of controls and 4 rats in each group of fasted, x-irradiated animals. Means and stand. errors of the means are plotted. Liver glycogen was determined by method of Kemp(10).

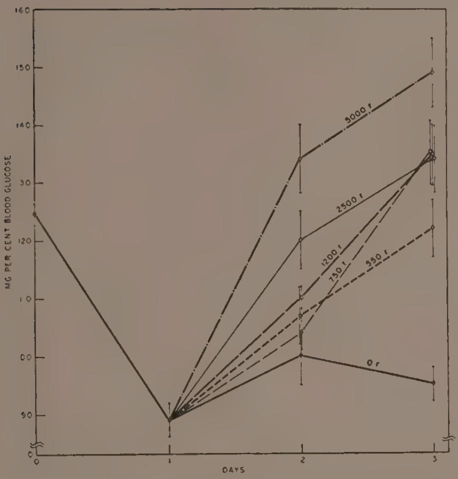

FIG. 3 (Exp. 3). Blood sugar levels as a function of time in control and fasted, x-irradiated rats. Fast was begun on day 0 and rats were exposed to x-radiations on day 1. Five rats were used in each group of controls and 4 rats in each group of fasted, x-irradiated animals. Means and stand. errors of means are plotted.

to or greater than that found in the normal, fed animal.

Blood sugar. From Fig. 2 and 3 it can be seen that during a 72-hour fast the blood sugar level drops by about 20% during the first 24 hours, rises slightly during the next 24 hours and remains at this new level over the final 24 hours. On the other hand, within 24 hours after exposure to x-irradiation the blood sugar level of fasting, x-irradiated rat increases and, at every dose (with the exception of 1200 r in Exp. 2), it continues to increase during the next 24 hours. Forty-eight hours after exposure to x-irradiation, concentration of sugar in blood of irradiated rats exceeds that found in normal, fed rats. There appears to be no clear relationship between the x-ray dose and blood sugar level. However, in each experiment the highest blood sugar levels, at both 24 and 48 hours after irradiation, are found in rats exposed to the highest dose (5000 r).

Discussion. The data presented indicates that exposure of fasting rats to whole-body x-irradiation results in an increase in liver glycogen and blood sugar levels. If the increased glycogen levels are a result of increased gluconeogenesis, then it is obvious that x-irradiation must cause appropriate glucose precursors to be made available. That glucose precursors are made available from mobilized protein is indicated by the finding of increased urinary excretion of urea in fasted x-irradiated rat(12). However, it appears that the availability of glucose precursors is not the only factor that determines amount of glycogen found in the liver following irradiation. This is indicated by the fact that an increased dose of x-irradiation over the range of 550 to 5000 r results in increased liver glycogen, while daily excretion of urea, the major end product of protein metabolism, is not altered by increasing the dose over the range of 500 to 2500 r(12). Furthermore, since it has been found that hypophysectomy prevents deposition of glycogen in the liver of irradiated rat(3), it seems that stimulation of the pituitary-adrenal system is necessary for the conversion of glucose precursors to liver glycogen. Since glycogen deposition increases with increasing dose, whereas availability of glucose precursors apparently does not, it seems possible that, once glucose precursors are available, the amount of glycogen deposited in the liver is determined by strength of stimulus delivered to the pituitary-adrenal system.

The fact that the fasted, x-irradiated rat is hyperglycemic clearly shows that x-irradiation does not, as was concluded by McKee(4), merely prevent the fall of blood sugar due to fasting. The hyperglycemia of the x-irradiated rat does not appear to be necessarily related to the accumulation of glycogen in the liver. This is indicated by the fact that, at all doses, blood sugar level of irradiated rat continues to rise 48 hours after irradiation, whereas (with the exception of the rats exposed to 5000 r) maximum levels of liver glycogen are obtained within 24 hours.

Summary. 1. Whole-body x-irradiation increases the percentage of liver glycogen in the fasting rat. At 24 hours after time of irradiation glycogen deposition was shown to increase with an increase in dose. 2. In rats exposed to 5000 r the percentage of liver glycogen was greater 48 hours after the time of irradia-

tion than at 24 hours. In animals exposed to 550, 750, 1200 or 2500 r the percentage of liver glycogen was not greatly different at 48 hours and 24 hours after the time of irradiation. In no case did the liver glycogen levels in the irradiated rats equal or exceed that found in normal, fed rats. 3. Whole-body x-irradiation increased the blood sugar levels in fasting rats. Larger increases were noted at 48 hours after the time of irradiation than at 24 hours. At 48 hours after the time of irradiation the blood sugar level was, in every case, greater than that of fed, normal rats.

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A Winter Isolation of Western Equine Encephalitis Virus from Hibernating *Culex tarsalis* Coquillett. (22193)

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The means of survival of Western equine encephalomyelitis virus (WEE) during the winter months has been a perplexing question since the disease was first recognized. Several explanations have been proposed for reintroduction or reappearance of the virus every year in certain endemic areas. The 3 most common theories are: 1. Migrating birds bring the virus northward each spring. 2. Arthropods, other than mosquitoes, harbor the virus during winter and infect susceptible birds during the most active stages of the arthropod life cycle. 3. Infected mosquitoes hibernate over the winter and infect susceptible birds and mammals during the following spring. It is the purpose here to present our findings and discuss the last possibility. As pointed out by Eklund(1,2) the conditions under which a mosquito-vertebrate cycle fails to account for maintenance of virus must be better understood before alternative mechanisms can be explored intelligently.

During the summer months WEE virus has been recovered from *Culex tarsalis* Coquillett frequently and from other mosquitoes occasionally in endemic areas(3-8). Hibernating *C. tarsalis* have not previously been reported in such numbers, and few of these have been tested for the presence of WEE virus. Although winter isolations of WEE virus from this species have been made in California during November and January(9) it is felt that these mosquitoes did not represent true hibernators. We consider the following factors to be indicative of true hibernation: the presence of fat bodies, lack of feeding activity of mosquitoes, absence of male mosquitoes, quiescence of female mosquitoes, and temperatures prohibitive for mosquito survival outside of the hibernating sites.

Methods. Mosquito hibernation sites. C. tarsalis mosquitoes were found hibernating in abandoned mines during winter months. These mines were in the foothills in Boulder and Larimer Counties, Colo., between elevations of 5000 and 8000 feet above sea level. The temperature from Dec. 22 through Feb. 20, was between 12.2°-13.3°C in a mine near

TABLE I. Hibernating *Culex tarsalis* Mosquitoes Collected in Colorado during the Winter of 1953-1954.

Date collected	Date tested	No. mosquitoes	No. pools	From	Location
12/15/53	1/19/54	38	1	6 mines	7 miles W of Masonville
30	"	587	12	3 "	3 " E " Jamestown*
"	"	1	1	1 mine	7 " W " Boulder
1/ 7/54	"	612	12	1 "	3 " E " Jamestown
13	2/16	1	1	1 "	1 " NW " Sunshine Canyon
14	22	63	1	Mines in vicinity of Jamestown	
21	16	3	1	" " " " Central City	
22	22	41	1	5 coal mines	12 miles NE of Fort Collins
2/ 9	"	15	1	1 mine	2 miles E of Masonville
Total		1361	31		

* Isolation made from one pool of 50 mosquitoes.

Masonville, and relative humidity in this mine fluctuated from 30 to 80%.* However, during the latter part of February, after a long dry period, the relative humidity dropped to 15%. No mosquitoes were found in this mine after this sharp drop. The mine shaft entrances varied from 4 to 6 feet in diameter; the shafts proper extended from 120 feet straight back from the entrances to side shafts which extended into unexplored depths. *Collection of mosquitoes.* From Dec. 15, 1953 to Feb. 9, 1954, mosquitoes (*C. tarsalis*) were collected at random from the abandoned mines. The mosquitoes were resting approximately 10 to 50 feet from mine entrances. In no cases where mine shaft was absolutely dark during daylight hours were any mosquitoes found. A few mosquitoes were found in mines after the date in spring when presence of active adult *C. tarsalis* in the open indicated that they were leaving hibernation sites. Collections were made by locating mosquitoes on rock surfaces with a flashlight and aspirating them with a hand aspirator. The mosquitoes were taken to the laboratory in small cages, identified, then stored at –20°C in 10 x 75 mm tubes until tested for virus. *Processing of mosquitoes.* The mosquitoes were ground by chilled mortar and pestle in inactivated 20% normal horse serum (pH 7.4). A volume of 2 ml of diluent was used for pools of 25 to 75 mosquitoes, and 1 ml for those pools of less than 25 mosquitoes. The suspensions were centrifuged 10 min. at 1000 x G at room temperature. Three-tenths ml of buffered saline (pH 7.4) containing 2 mg of streptomycin sulfate and 1000 units of penicillin G sodium were added to 1 ml of supernatant materials and the mixtures incubated overnight at 4°C. Each antibiotic-treated supernate was inoculated subcutaneously in 0.03 ml amounts into 4 freshly hatched chicks, which were then observed for a 72-hour period(10). The remainder of supernatant material was ampuled, then frozen in alcohol and dry ice and stored at –70°C for reference.

Results. A total of 1361 female *C. tarsalis* mosquitoes, as shown in Table I, was collected and made into 31 pools. One pool of 50 mosquitoes collected on Dec. 30, 1953, killed 4 out of 4 chicks within 24 hours. None of the other pools killed chicks. The brains of the 4 chicks showed no evidence of bacterial contamination when cultured in thioglycollate medium. Upon passage of the 20% brain suspension, 3 of 4 chicks died in 24 hours with no evidence of bacterial contamination.

The brains of these chicks, as well as the original infected mosquito suspension, were sent in a frozen state to the Communicable Disease Center, Virus and Rickettsia Section, Montgomery, Ala., where the agent was confirmed as WEE virus by complement-fixation tests and serum neutralization tests in mice.

Discussion. The isolation of WEE virus from hibernating *C. tarsalis* mosquitoes collected in December shows this species to be capable of harboring the virus through a period of adverse weather conditions. Although the isolation does not prove that *C. tarsalis* could carry enough virus through an entire winter to infect birds and mammals in the spring, it furnishes the best supportive evidence for this theory that has yet been obtained. The ratio of positive pools to total pools tested of hibernating mosquitoes is not incompatible with the results of summer collecting and testing.

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* As measured by a recording hygrothermograph manufactured by Instruments Corp. of Baltimore, Md.

Congregation of *C. tarsalis* in certain mines is noteworthy, and relatively large numbers of mosquitoes found in these mines suggests that temperature and relative humidity therein are tolerable for mosquito hibernation. Although mines are artificial hibernating sites, and it is obvious that the majority of individuals of this widespread species must hibernate in locations other than mines, conditions found in these mines may furnish clues as to types of natural mosquito hibernation sites.

Summary. A total of 1361 hibernating female *C. tarsalis* mosquitoes, comprising 31 pools, was collected in mines in Colorado foothills from Dec. 15, 1953, to Feb. 29, 1954. One of these pools, collected Dec. 30, 1953, contained WEE virus upon inoculation into freshly hatched chicks. The finding of such large numbers of hibernating *C. tarsalis* and the recovery of WEE virus from them, may contribute to the knowledge of the overwintering mechanism of this virus.

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Recent Isolations of Encephalomyocarditis Virus. (22194)

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Strains of encephalomyocarditis (EMC) virus have been isolated on several occasions. The earliest of these isolations was made by Jungeblut during poliomyelitis investigations, first from cotton rats in 1940 (Columbia-SK strain)(1) and from hamsters in 1943 (MM strain)(2), but these were initially regarded as strains of poliovirus. A virus producing encephalomyocarditis in animals, and thus named encephalomyocarditis (EMC) virus, was isolated in 1945 by Helwig and Schmidt from a chimpanzee dying of myocarditis at Dania, Fla.(3,4). This virus was later found to be immunologically identical to Columbia-SK and MM viruses(5). In 1948 Mengo encephalomyelitis virus was isolated at Entebbe, Africa, from a rhesus monkey(6) and this virus was similar or related to the EMC virus. Since then similar types of virus have been isolated in Africa from man, mosquitoes, and monkeys(7). The present report describes 3 more isolations of viruses belonging to the EMC group.

On June 12, 1952, specimens of lung, liver, spleen, kidney and stomach contents from a mandrill baboon which had died of unknown cause at the Anthropoid Ape Research Foundation Farm in Dania, Fla., were received at the Communicable Disease Center from the Miami Regional Laboratory of the Florida State Board of Health for study. The baboon had died 24 hours after the first visible signs of illness. All that was noted by the veterinarian in charge during this 24-hour period was that the baboon could not stand and refused food. The animal had been at the Foundation farm in Florida for 3 or 4 years.

Methods. The liver and spleen were pooled as were kidney and lungs. Ten per cent suspensions of these pools and the stomach contents were prepared and, after addition of suitable amounts of penicillin and streptomycin, each of the 3 suspensions was inoculated intracerebrally into groups of 5 mice. All inoculated mice died between the 4th and 14th days. Cultures of the brains revealed bacterial contamination. These brains were inoculated into a second group of mice which died on 3rd to 6th day. No evidence of bacterial contamination was observed when brains of these mice were cultured on blood agar and in thioglycollate broth. A suspension of 2 of these 2nd mouse passage brains had a mouse intracerebral LD_{50} titer of $10^{6.3}$. The titer of 3rd and 4th mouse passage material was $10^{6.3}$ to $10^{6.8}$.

Results. Neutralization tests were carried out by incubating mixtures of 10-fold dilutions of the agent and undiluted antiserums to various viruses at 4°C overnight and then inoculating each of the mixtures intracerebrally into 5 mice. Neutralization was not considered positive unless the LD_{50} endpoint of test serum was 1.5 logs lower than normal serum control. The agent was not neutralized by antisera to eastern or western equine encephalomyelitis or St. Louis encephalitis viruses, but was neutralized by EMC antiserum. Antiserums to EMC virus and the new agent were prepared by a single subcutaneous inoculation of guinea pigs with live material. Reciprocal neutralization tests were made with these serums. Both EMC virus and the new agent were neutralized to essentially the same degree by heterologous as well as homologous antiserum.

A second isolation of EMC virus was made from a monkey brain submitted by the Florida State Health Department on Feb. 9, 1953. The virus was also isolated from brain of a mouse which had been inoculated at the Florida State Health Department Laboratory with brain material from this same monkey. This monkey had been housed at the same farm in Dania, Florida, where the baboon mentioned earlier had been kept. The monkey species and its clinical history were not provided with the specimen and could not be obtained later. A suspension of the brain of this monkey, upon inoculation into mice, yielded a virus which behaved essentially like that isolated earlier from the baboon.

EMC virus was isolated again from a squirrel brain submitted by the Florida State Health Department Laboratory on Feb. 11, 1954. This squirrel, which originated in Palm Beach County, Fla., had appeared sick and bit a child who handled it. Therefore, it was captured to have it examined for evidence of rabies infection. As far as we are aware, this inadvertent finding represents the first isolation of EMC virus from a wild animal indigenous to North America, although antibodies to the EMC group of viruses has been previously demonstrated in wild rats in the southern United States by Warren and coworkers(8).

The second mouse passage material of the baboon strain was titrated in 3-week-old mice by the subcutaneous, intramuscular, intraperitoneal, intranasal, and intracerebral routes. An inoculum of 0.03 ml brain suspension material was used. The agent titered about 10-fold higher by the intracerebral than by the intramuscular, subcutaneous, and intraperitoneal routes, and about 1000-fold higher than by the intranasal route. The incubation periods or symptoms did not vary significantly by the various routes. Mice receiving the lower dilutions died in 3 to 5 days after inoculation, while death from the more dilute suspensions could be delayed up to the 10th day. Those mice receiving the low dilutions of brain suspension became weak, with ruffled hair coats, and they would be found dead the day after these symptoms appeared. Occasionally convulsions would be seen. Mice receiving higher dilutions would first be seen walking with an unsteady gait. This would be followed by a complete paralysis of the hind legs. These symptoms would continue for one to 4 days and then be followed by prostration and death.

Cotton rats inoculated intranasally or subcutaneously with a 10^{-3} dilution of virus became weak and died within 48 to 72 hours after inoculation, while guinea pigs displayed a febrile response only and survived subcutaneous inoculation.

The lesions in inoculated mice consisted of glial nodules scattered through the corpus callosum, internal capsule, thalamus and midbrain. There was almost complete destruction of the Purkinje cells of the cerebellum. The cerebral cortex was essentially free of lesions. The lumbar portions of the spinal cord showed an occasional necrotic neuron in the ventral gray horn. Demyelinization was present in certain fiber tracts and nerve trunks. The heart showed interstitial round cell infiltration and mild fiber fragmentation in the auricular myocardium. The spleen, lungs, liver, gut, and kidneys were free of demonstrable lesions.

Summary. During the past 3 years encephalomyocarditis virus was isolated twice from captive primates imported to Florida and once from a wild squirrel originating in Florida.

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Effect of Temperature on Activity of Guinea Pig Complement. (22195)

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When human complement (hu C′) and sensitized sheep erythrocytes (EA) interact, both extent of lysis and shape of the lytic curves obtained are markedly dependent on temperature(1). These findings have recently been explained(2) on the basis of proportionality between temperature and rates of formation and decay of an intermediate complex $EAhuC'_A$.

Since the great majority of both experimental and routine laboratories have used guinea pig (g.p.) rather than hu C′ in their investigations, and since the choice of 37°C as reaction temperature appeared to be quite arbitrary, the effect of temperature on lysis by g.p.C′ was investigated. These studies were greatly facilitated by data from several laboratories(3,4) on EAg.p.C′1,4,2, the complex between EA and g.p.C′1, C′4 and C′2.

Methods. Sheep blood in 500 ml lots was preserved with the addition of 2000000 units of penicillin "G" and 25 ml of 0.15 *M* Trisodium ethylenediamine tetraacetic acid (Na_3E.D.T.A.)(4). Veronal buffer(5) containing 5×10^{-4} *M* Mg^{++} and 1.5×10^{-4} *M* Ca^{++} was used for all dilutions and washings. Pooled guinea pig sera, stored in small lots at –25°C, were the source of g.p.C′. Kinetic analyses were carried out according to the technic of Mayer and Levine(6). Bath temperatures were held constant to ± 0.1°C. All solutions were equilibrated at the temperature of the experiment before mixing.

Results. Two flasks, each containing 10 ml of EA, 10 ml of buffer and 5 ml of g.p.C′/170 were incubated at 37°C, and 27°C respectively. After sampling was completed, 2 more flasks were run at 37°C and 32°C. The reaction curves are plotted in Fig. 1. The extent of reaction at 37°C was considerably less than at either 27°C or 32°C.

The next experiment was designed to study

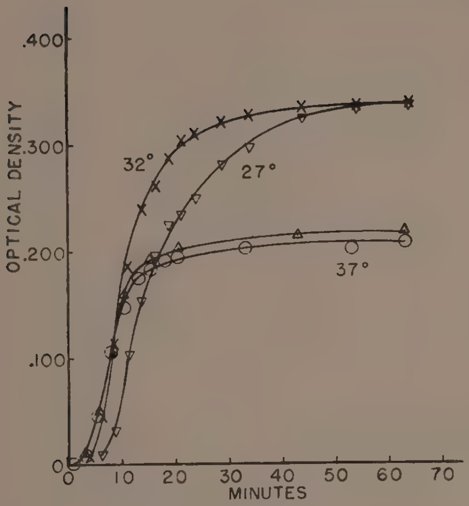

FIG. 1. Effect of temperature on lysis by a constant volume of g.p.C′.

the effect of temperature on formation of the intermediate complex EAg.p.C′1,4,2. The complex was prepared by the method of Leon *et al.*(4), using a sublytic dilution of g.p.C′. Flasks containing 30 ml of cells, 30 ml of buffer and 15 ml of g.p.C′/540 were incubated at 37°C, and 27°C and later the same day at 37°C and 32°C. At intervals 3 ml samples were withdrawn, mixed with 0.5 ml of g.p.C′/40 and 0.2 ml of 0.15 *M* Na$_3$E.D.T.A., stoppered and incubated at 32°C for one hour. Na$_3$E.D.T.A. binds Ca^{++} and Mg^{++} and therefore prevents further reaction of any complement components except for C′3(4,7). The C′3 present in the g.p.C′/40 will lyse only those cells that have reached the stage EAg.p.C′1,4,2, through reaction of EA with g.p.C′/540. The results are plotted in Fig. 2.

Discussion. In the presence of a constant amount of C′3 the extent of lysis is proportional to the quantity of EAg.p.C′1,4,2 available(4,8). The quantity of EAg.p.C′1,4,2 available will, in turn, be a function of the quantity of C′1, C′4 and C′2 present and of the reaction temperature (Fig. 2). Thus, at 27°C and 32°C, where the concentration of EAg.p.C′1,4,2 is *maintained* at a high level for a considerable length of time, more lysis is obtained than at 37°C where the concentration of intermediate is smaller and its stability lower.

In this laboratory, where both hu C′ and g.p.C′ are used, the majority of lytic reactions are now carried out at 32°C, the temperature previously shown to be optimal for hu C′(1).

Summary. Guinea pig complement is considerably more reactive at 27°C and 32°C than at 37°C. These findings have been ex-

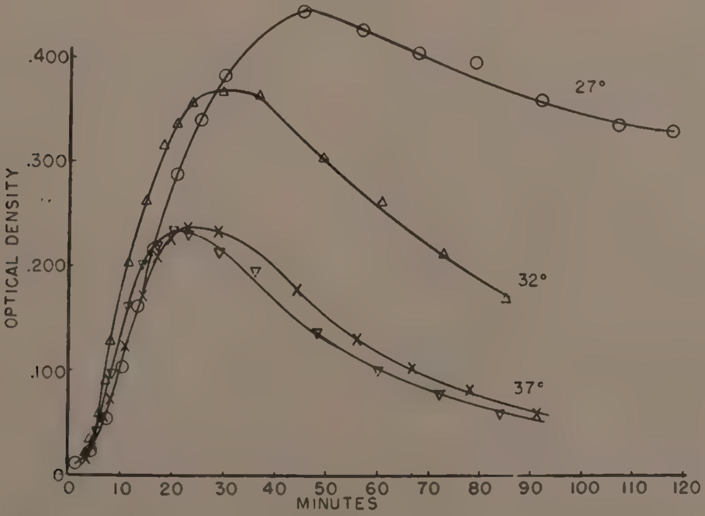

FIG. 2. Effect of temperature on formation and decay of EAg.p.C′1,4,2.

plained by differences in the maximal formation and the stability of an intermediate complex, EAg.p.C′1,4,2.

The author wishes to express his appreciation for the technical assistance of Miss Edna Chapman.

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Effect of X-Irradiation and Hypophysectomy on Globule Leucocytes In Rat Intestine.* (22196)

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Globule leucocytes are distributed widely in urinary(1), respiratory(2) and digestive mucous membranes (Fig. 1) of the rat. They are distinguished by presence in their cytoplasm of abundant, coarse, metachromatic acidophilic inclusions and by the fact that they occur only in the epithelium and tunica propria. Cytological evidence indicates that they arise by direct transformation from lymphocytes(3). Like lymphocytes, they are reduced in number by treatment with corticotropin or cortisone(2). Because of these observations and the well-known radiosensitivity of lymphocytes, this study was made of the response of globule leucocytes to total body x-irradiation. Observations are reported also on effect of hypophysectomy.

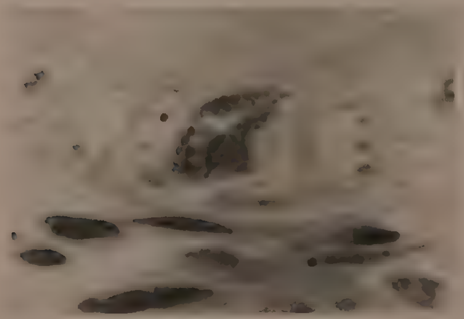

FIG. 1. Globule leucocyte in epithelium of duodenal crypt of hypophysectomized rat. ×1450.

Procedure. Female Sprague-Dawley rats weighing 150-190 g were used. During early experiments, control rats were pair fed against irradiated animals. Since it was observed that reduction in food intake was without effect, the animals were fed *ad libitum* in the final experiments. Diet consisted of Purina Laboratory Chow supplemented twice weekly with greens, oranges, and a vitamin concentrate. Temperature of animal quarters was maintained at 75 ± 3°F. All irradiated animals (except as noted below with relation to shielding experiments) were exposed to whole body irradiation while held in flat cylindrical holder constructed of ¼-inch sheet Lucite and radially divided into 9 equal compartments. Radiation was administered by Keleket Theron X-ray therapy machine operated at 200 KV with ½ mm Cu and 1 mm Al filtration. Distance from center line of x-ray tube to table on which animal container rested was 110 cm. Dose rate was indicated by Victoreen rate meter and was held constant at about 11 r/min by varying x-ray tube cur-

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TABLE I. Effect of X-Irradiation on Globule Leucocytes in the Ileum.

Days after irradiation	No. rats	X-ray dose (r)	Mean No. globule leucocytes/section	P values*
0	20	—	251.3 ± 104.6†	
½	2	650	84.0 ± 15.3	<.001
¾	1	"	22.5	
1	4	"	19.2 ± 8.0	<.001
2	2	125	92.0 ± 13.5	"
2	3	650	7.8 ± 1.3	"
4	5	"	2.6 ± 1.2	"
5	2	"	2.5 ± 1.0	"
6	2	"	5.3 ± 2.3	"
7	5	"	6.5 ± 4.5	"
14	4	"	13.8 ± 1.2	"
21	2	"	21.5 ± 11.0	"
30	2	"	71.2 ± 6.5	"
35	2	"	201.5 ± 32.5	NS
43	2	"	220.4 ± 21.6	"
53	2	"	257.8 ± 8.8	"

NS = Not significant ($P > .05$).

* Counts compared with those for the rats which received no irradiation.

† Stand. dev. = $\sqrt{\frac{\Sigma x^2 - \bar{x}^2}{n}}$.

rent within range of 20-25 ma. Output was calibrated by giving a timed exposure to Victoreen r-meter chamber inserted within animal chamber in a position receiving average radiation intensity. At autopsy, samples of ileum were fixed in Helly's fluid. Paraffin sections were cut at 5 μ and stained in phosphotungstic acid hematoxylin and basic fuchsin. Globule leucocytes were counted in 3 non-adjacent sections from each animal and these counts averaged. The number of animals sacrificed at various time intervals after irradiation and radiation doses employed are summarized in Table I. To determine whether x-irradiation acted directly or indirectly on globule leucocytes, additional animals were divided into 3 groups: a. those having a segment of intestine exteriorized and protected by lead shield during irradiation; b. those treated like group a, but with shield made of paraffin; c. those treated like group a, but without exposure to x-irradiation. Animals were anesthetized with subcutaneous injections of Nembutal (0.04 or more mg/g body weight), and a loop of small intestine was exteriorized through incision in the flank. In initial experiments, 4 rats were irradiated simultaneously while fastened to plywood square with their ventral sides up and shield toward center of board. They were arranged symmetrically with relation to the x-ray beam. For experiments at 600 r and 800 r, smaller shields were used to permit irradiating anesthetized animals in the regular Lucite holder instead of tying them down as in earlier experiments. The intestinal loop was anchored in depths of the shield by a loose stay suture passed around the intestine (through the mesentery) and around a wire hook at end of shield. The shield itself was fastened to skin of the animal by sutures through wire loops at each end of the shield. These groups were irradiated at 20 r/min by a Pikens X-ray machine operated at 235 KV. In all shielding experiments, one limb of the shielded segment of intestine was marked at the entrance to the shield with a mattress suture with knotted end pointing toward shielded loop. At autopsy, small samples of shielded and non-shielded intestine were taken approximately 1.5 cm on each side of this suture. The procedure for preparation and study of these tissues was the same as for those from non-shielded animals. Experiments on hypophysectomized animals were performed in 8th or 9th post-operative week. In all cases, completeness of operation was established by microscopic examination of serial sections of the pituitary capsule. All incompletely hypophysectomized animals were eliminated.

Observations. In non-hypophysectomized rats there was a statistically significant reduction in number of intestinal globule leucocytes within 12 hours after irradiation, with a 99% disappearance of cells by 4th and 5th days after irradiation with 650 r (Table I). Beginning about the 6th day after irradiation and continuing at a relatively slow rate until about the 30th day, globule leucocytes increased in number. During 4th week after irradiation, reappearance of cells accelerated and by 35 days their number was within the normal range. Since globule leucocytes arise within the tunica propria from small lymphocytes(3), which are radiosensitive, it is pertinent to note that rate of increase in absolute lymphocyte count in the circulation(4)

TABLE II. Effect of X-Irradiation and Shielding on Globule Leucocytes in the Ileum.

Exp.	Type of shield	Dose (r)	Days after irradiation	No. rats	Mean No. globule leucocytes/section	P values
I		0	1, 4, 7	6	170 ± 50 *	NS †
	Lead	0	1, 4, 7	6	215 ± 40	" †‡
II		650	2, 4, 7	13	5.6 ± 2.3	<.001†
	Paraffin	"	2, 4, 7	3	4.4 ± 4.3	NS ‡
	Lead	"	2, 4, 7	6	286.7 ± 44.4	" †
III		800	3	3	5.9 ± 3	<.001†
	Paraffin	"	3	1	9.7	
	Lead	"	3	2	188.2 ± 20.9	NS †
IV		"	4	11	1.6 ± 1.9	<.001†
	Paraffin	"	4	1	4	
	Lead	"	4	9	148.4 ± 42.8	" †
V		0	0	11	109.8 ± 63.4	" †
Hyp.		600	4	8	10.9 ± 9.4	NS §
	Lead	"	4	6	100.6 ± 85.7	" ‖

* Stand. dev. = $\sqrt{\frac{\Sigma x^2 \cdot x^2}{n}}$. † Compared with non-hypophysectomized, non-irradiated rats (Table I). ‡ Compared with non-shielded animals of same experiment. § Compared with hypophysectomized, non-irradiated rats (Exp. V). ‖ Compared with non-hypophysectomized rats 4 days after irradiation with 650 r (Table I).

Hyp. = Hypophysectomized. NS = Not significant (P >.05).

roughly parallels recovery of globule leucocytes. The extended period of slow recovery, therefore, may be interpreted as being due to scarcity of parent cells from which new globule leucocytes may be formed.

The effect of radiation on globule leucocytes is primarily a direct one as shown by shielding experiments (Table II). Segments of intestine which were either unshielded or paraffin-shielded showed the same reduction in number of globule leucocytes as in irradiated animals not subjected to shielding procedure. In contrast, samples of small intestine which had been lead-shielded during irradiation showed no reduction at 650 r, the LD_{20} 30 days(5). At 800 r, the LD_{100} 30 days(5), only 40% reduction from normal occurred. X-irradiation may act directly on globule leucocytes themselves or indirectly through changes elicited in their tissue environment. The latter possibility must be considered in view of local disappearance of these cells in areas of tracheal inflammation.

It is probable that radiation-induced stress is responsible for partial disappearance of these cells in lead-shielded segments of intestine of non-hypophysectomized rats receiving 800 r. In the absence of the hypophysis, the comparable LD_{100} 30-day dosage for hypophysectomized rats (600 r) failed to induce a significant reduction in number of globule leucocytes in lead-shielded segments of the intestine (Table II, Exp. V). The shielding procedure was not involved, since in non-irradiated animals, neither shielded nor unshielded segments of intestine showed a statistically significant change in population of globule leucocytes. Neither was it likely to be due to radiations which penetrated the shield, since the theoretical protection afforded by shields was in the neighborhood of 99.5%. Finally, we have demonstrated that treatment with corticotropin or cortisone reduces the number of globule leucocytes(2).

Hypophysectomy, without irradiation, caused a marked reduction in number of globule leucocytes just as it decreases thymic and splenic weight from that of normal rats (6). After x-irradiation of hypophysectomized rat, the number of globule leucocytes was reduced to essentially the same level as in irradiated non-hypophysectomized rat (Table II). The percentage of reduction, however, was significantly less than that of non-hypophysectomized animals. Hypophysectomy also caused a shift in position of a considerable portion of the globule leucocytes from the tunica propria (where the largest percentage occurs in non-hypophysectomized rat) to the crypt epithelium.

Summary. Within 4 days after whole body x-irradiation (650 r), globule leucocytes were reduced to 1-2% of their normal number in the intestine of the rat. Reestablishment of normal numbers of cells began toward end of the first week after irradiation and was completed by the 5th week. The effect of irradiation on globule leucocytes was chiefly a local one, being prevented by shielding the intestine with lead during irradiation. Only with the LD_{100} 30 days dosage (800 r) was there a significant reduction in number of globule leucocytes in lead-shielded intestinal segments. This appeared to be the indirect result of stress induced by irradiation, since the effect was prevented by hypophysectomy. Hypophysectomy alone reduced markedly the number of intestinal globule leucocytes.

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Strontium^{85} Metabolism in Man and Effect of Calcium on Strontium Excretion.* (22197)

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Sr^{90}, a β emitting isotope with a 25 year half life is one of the major hazards of nuclear fission because of its selective localization in the skeleton. Its entry into the human body and its pathways of elimination are, therefore, of great concern. Availability of a carrier-free γ emitting isotope of strontium, Sr^{85}, with a half life of 65 days, facilitated this investigation of the metabolism of radiostrontium in man(1). Data on metabolism of intravenously administered Sr^{85} are reported in this communication.[‡]

Methods. Carrier free Sr^{85}, 0.1-0.4 μc/kg body weight was injected intravenously in 4 human subjects. Blood samples and urine were obtained at 1, 4, 8 and 24 hour intervals initially. Subsequently, urine, stool and plasma were analyzed daily. Radioactivity was determined in 1-5 ml plasma and urine and on stool samples weighing up to 5 g. Significant counts on these samples were obtained up to 24 days. A Nuclear scintillation well counter, Model DS3 was used: through special lead shielding the background was reduced to 140-180 CPM.

Results. Fig. 1 shows plasma levels, urinary and fecal excretions of Sr^{85} of a 72 year

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[*] This study was performed under Contract AT(30-1)-1763 between Montefiore Hospital and the U. S. Atomic Energy Commission.

[†] Trainee, National Cancer Institute.

1. Laszlo, D., Spencer, H., Brothers, M., Berger, E., and Hart, H. E., Int. Conf. Peaceful Uses Atomic Energy, Geneva, June 1955.

[‡] Produced in Oak Ridge National Laboratory Cyclotron by the nuclear reaction of Rb^{85} (p,n) Sr^{85}. Radionucleides of Sr^{85} were isolated from the target material, Rb_2CO_3, and other impurities with no strontium carrier added. This separation was performed by H. T. Russell, Radioisotope Operations Division of Oak Ridge National Laboratory. We thank these laboratories for their cooperation.

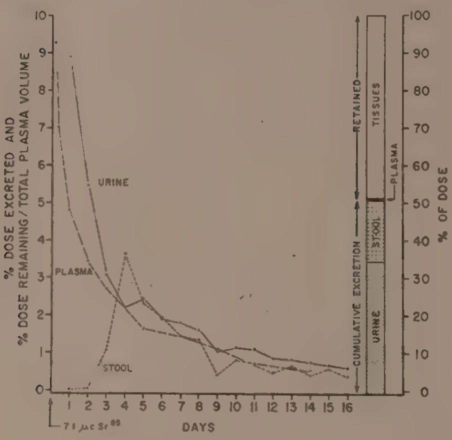

FIG. 1. Intravenous tracer dose of Sr^{85}; plasma levels, urinary and fecal excretions.

old ambulatory female with osteoporosis. (Case 3, Table I). Initially, plasma levels and urinary excretions declined rapidly, fecal Sr^{85} content was highest on 4th day and from there on plasma levels, fecal and urinary Sr^{85} excretions declined gradually with the lines falling closely together. In 16 days a total of 34.5% was excreted in the urine, 16.3% in the stool; 0.5% was present in the total plasma volume and 48.7% of the dose was retained in the tissues.

Similar experiments were performed on 3 other subjects. Plasma levels and urinary excretions of the first 48 hours of the studies are listed in Table I. Patients 1 and 4 had no skeletal disease. Patient 2 had Paget's disease of the bone. Significant differences in plasma levels and urinary excretions were noted in the 4 subjects. The 48 hour urinary strontium excretion ranged from 6.5-28.3%. The latter was excreted by pt. 4 who eliminated 57.2% in 15 days in the urine and 11.0% in stool.

After radioactivity of the samples declined to low levels, a second tracer dose of Sr^{85} was injected to each patient at the midpoint of a 2 hour infusion of calcium gluconate, containing approximately 1 g Ca^{++}. The marked enhancement of urinary strontium excretion is shown in column B, Table I. A 5-15 fold increment is noted at one hour. The enhancement continued for several hours after termination of the calcium infusion and was followed by a temporary depression on the next 2 to 3 days. In spite of the marked increase of urinary Sr^{85} excretion, the plasma levels were only slightly different and fecal Sr^{85} excretions were not enhanced. The magnitude of the calcium effect upon urinary strontium excretion becomes evident by comparing the cumulative 24 hour Sr^{85} excretions following administration of the isotope with and without calcium in the 4 subjects: 4.40 vs 21.5%, 4.2 vs 13.7%, 8.9 vs 18.2%, and 18.6 vs 29.5% respectively.

To study whether strontium excretion is similarly enhanced if both the tracer and calcium are injected slowly, the 2 experiments listed

TABLE I. Effect of Intravenous Calcium on the Urinary Excretions and Plasma Levels of Sr^{85}.

	1 hr		4 hr		8 hr		24 hr		48 hr	
Case No.	A	B	A	B	A	B	A	B	A	B
1	.40 (10.49)	6.14 (12.02)	.63 (7.40)	5.82 (7.91)	.76 (6.54)	4.45 (6.61)	2.61 (4.69)	5.13 (4.69)	2.56 (3.48)	1.69 (3.54)
2	.41 (7.37)	4.52 (7.77)	.44 (4.26)	3.70 (4.37)	1.26 (3.42)	2.89 (2.95)	2.05 (2.19)	2.58 (2.27)	2.38 (1.69)	1.19 (1.65)
3	1.17 (12.50)	6.03 (13.62)	.96 (9.26)	4.95 (8.88)	1.26 (7.01)	2.22 (6.93)	5.52 (4.80)	5.01 (4.38)	5.48 (3.43)	3.51 (3.50)
4a	1.38 (10.36)	7.85 (9.01)	3.13 (7.08)	7.19 (5.99)	4.36 (5.08)	6.68 (4.61)	9.80 (3.33)	7.80 (2.80)	9.61 (2.16)	6.16 (2.02)
4b	.09 (1.65)	.11 (1.14)	.87 (5.07)	2.78 (4.85)	5.34 (5.50)	11.00 (5.63)	7.53 (3.60)	9.52 (2.86)	8.95 (2.04)	4.78 (1.65)

Figures represent Sr^{85} in % dose excreted from 0-1 hr, 1-4 hr, 4-8 hr, 8-24 hr, 24-48 hr; % dose of total plasma volume in parentheses.

A = experiments without calcium; B = with calcium, see text.

Exp. 4a and 4b were performed on the same patient; in 4b Sr^{85} was infused in 5 hr without calcium (A) and with calcium (B).

under 4b were carried out. The isotope was infused for 5 hours. The plasma level of Sr^{85} rose gradually and was 5.5% at 8 hours (column A): it declined thereafter at rates comparable to that of the rapid injection of Sr^{85}. Urinary strontium excretion increased gradually and was highest at 8 hours. The cumulative 24 hour urinary excretion was 13.8% (column A). The marked enhancement of strontium excretion by simultaneous but slow administration of the same dose of calcium, approximately 1 g Ca^{++}, infused for 1 hour preceding and for 5 hours simultaneously with Sr^{85} is shown in column B. The cumulative 24 hour urinary strontium excretion rose to 23.4%.

These experiments illustrate that the major route of excretion of intravenously administered strontium is via the kidney; in 15 days as much as 57% was excreted in one patient. Calcium significantly increases renal clearance of strontium and thereby decreases the body load.

Summary. Radiostrontium metabolism following intravenous administration of tracer doses of strontium^{85} was studied in 4 human subjects. Plasma levels, urinary and fecal strontium excretion were measured. The plasma levels declined rapidly. The main route of excretion is via the kidney. Rate of strontium excretion seems to depend upon the state of bone metabolism. It was low in Paget's disease and high in senile osteoporosis. Simultaneous administration of calcium gluconate significantly enhanced the strontium excretion.

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Mechanical Fragility of Erythrocytes from Patients with Paroxysmal Hemoglobinuria.* (22198)

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It has been established that pH is an important factor in *in vitro* hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH). *In vitro,* hemolysis of PNH erythrocytes in normal human serum is characterized by: 1) a hemolytic system which requires properdin, complement and Mg^{++}(4), all normal constituents of normal serum; 2) absence of abnormal hemolysins or agglutinins in patient's serum(2,3,4); 3) maximum hemolysis at pH 6.5-7.0; 4) minimal hemolysis in normal human serum outside pH range 6-8.

As pH of PNH blood is decreased from pH 7.8, and hemolysis becomes maximum, it is conceivable that the hemolytic system could weaken the erythrocyte cell wall in such manner that *in vivo* trauma would augment hemolysis. This question was submitted to test by determining mechanical fragility (MF) *in vitro,* of normal and PNH erythrocytes in active and inactive properdin systems at varied levels of pH.

Materials and methods. To determine the effect of decreased pH on MF, hydrochloric acid of N/1.2 to N/8 was used to obtain the desired pH of blood samples. To maintain this pH during rotation, imidazole in HCl (3.44 g imidazole/100 ml of 0.2 N HCl) was used as buffer because of its effectiveness when used in small quantities(7). Although this solution is hypertonic, it becomes approximately isotonic (isotonic equals 1.72 g/100 ml of 0.1 HCl), when it is diluted with equal volume of HCl used to alter pH. Defibrinated blood samples were obtained from 3 normal males, 3 normal females and 3 patients with PNH. Of the 3 PNH pa-

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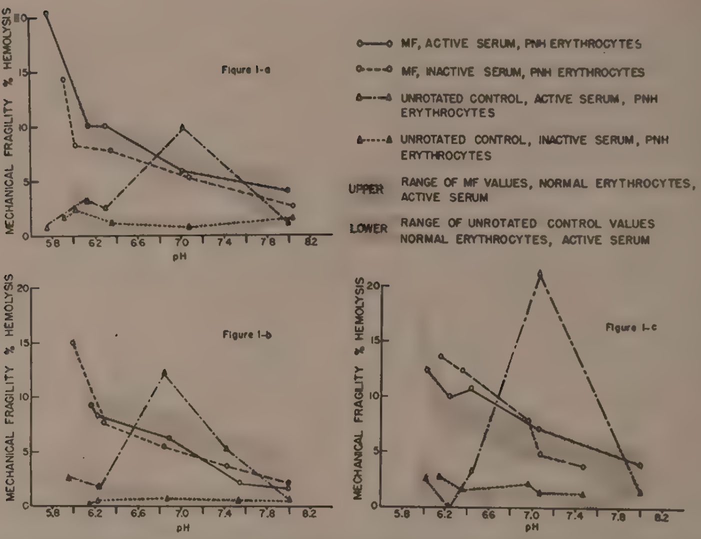

FIG. 1. Results of mechanical fragility studies done at various values of pH on blood of 3 patients with paroxysmal nocturnal hemoglobinuria (Fig. 1-a, 1-b, and 1-c correspond to Cases I, II, and III respectively) and 6 normal subjects.

tients, Cases I and III (Fig. 1-a and 1-c) had approximately 50% transfused cells at time of observation. The erythrocyte population of Case II (Fig. 1-b) contained no transfused cells at time of observation. Each sample was adjusted to 35% hematocrit and divided into aliquots (1.5 ml). The aliquots obtained from each PNH sample were divided into 2 groups, one group was designated the active group and the other inactive. Serum from each aliquot was removed and placed into corresponding tubes. Serums of inactive aliquots were heated in 60°C waterbath for 30 minutes to inactivate complement, thus destroying the activity of PNH hemolytic system, while the hemolytic system of active serum was allowed to remain intact. From the serum of each aliquot, 0.2 of the total volume was removed. This 0.2 volume was replaced by and thoroughly mixed with 0.1 volume of HCl and 0.1 volume of imidazole buffer to obtain the desired pH. The erythrocytes were resuspended in the acidified buffered serum and thoroughly mixed. A pH was determined on each sample in a Beckman pH meter (Model G) before and after the MF determination, the average of the two being used for interpretation of results. The MF of each aliquot was determined according to the method of Shen, Castle, and Fleming (9) as modified by Ham(1).

Results. Range of values of MF for normal erythrocytes in the pH range 5.95-8.0 is given in Table I and represented by the upper shaded area in Fig. 1. Range of percent hemolysis occurring in unrotated samples of normal erythrocytes subjected to a similar pH range is given in Table I and represented by the lower shaded area in Fig. 1. Normal values represented in Table I and on Fig. 1 were obtained by extrapolation of maximum and minimum values of MF at every 0.2 pH unit from a composite graph containing results of studies on 6 normal subjects.

Results of studies done on three PNH patients are presented in Fig. 1-a, 1-b, and 1-c. The curves labeled unrotated control, active serum, PNH erythrocytes in these Figures demonstrates the hemolysis of PNH erythrocytes in acidified serum. This observation indicates that the properdin system is intact in samples with active (unheated) serum. Correction for hemolysis in the rotated active sample attributable to the PNH mechanism

TABLE I. Range of Values of MF of Normal Erythrocytes at Various Values of pH.

pH	No. of observations	Mechanical fragility Hemolysis in % Range	Avg	Unrotated control Hemolysis in % Range	Avg
5.95	3	16.8 -19.2	17.5	0 -4.3	1.8
6.0	4	14.0 -15.9	14.6	1.0-5.6	1.9
6.1	4	8.3 -12.2	9.8	0 -4.1	1.4
6.2	6	7.1 -10.4	9.1	.5-2.8	1.2
6.4	6	7.1 - 9.8	8.5	.5-1.8	.9
6.6	6	5.7 - 8.8	7.6	0 -1.5	1.0
6.8	6	4.6 - 8.4	6.5	.6-1.5	1.1
7.0	6	4.3 - 8.2	6.4	.4- .9	.7
7.2	6	3.8 - 7.0	5.6	.4-1.2	.8
7.4	6	4.6 - 6.0	5.2	0 -1.1	.8
7.6	6	2.1 - 5.9	4.4	0 -1.1	.8
7.8	5	1.6 - 5.6	3.7	.6-1.6	1.0
8.0	4	1.70- 5.0	3.7	.6-1.6	1.3

was done by subtraction of unrotated active control value from the active rotated sample to obtain per-cent hemolysis attributable to mechanical trauma.

The reproductibility of the technic was tested by dividing a sample of normal blood into 7 aliquots and determining the MF of each separately after adding the same amount of acid and buffer. The range of pH values of the 7 aliquots was 6.9 to 7.05 and the range of MF values was 5.2-8.5%.

Morphologic evaluation of cell shape was done by examination of Wright stained erythrocytes. Spherocytes and budded forms were seen at pH 6 in both PNH and normal erythrocytes.

Discussion. It has been postulated that increased MF of erythrocytes may be associated with increased spheroidicity or firm agglutination of red blood cells(10). Increased spheroidicity was observed at pH 6, but no agglutination occurred in the studies. Spheroidicity may be explained as follows: With a decrease in intracellular pH, the amphoteric hemoglobin molecule shifts toward its undissociated state, each hemoglobin molecule releasing a maximum of 10 K^+. Since each K^+ acts as an osmotic particle, the osmotic gradient into the cell is increased. This is further augmented by the passage of HCO_3^- and Cl^- into the cell to neutralize the excess of impermeable K^+. Osmotic equilibrium is maintained by the passage of H_2O from the serum into cells thus increasing cell volume (8).

It has been proposed that erythrocytes of congenital spherocytosis, sickle cell anemia and acquired hemolytic anemia may be conditioned in the spleen in such manner as to increase both osmotic and mechanical fragility(5). Perhaps pH is one factor in the "conditioning" process taking place in the spleen. Studies of pH in splenic circulation are not at present available. However, Watson and Paine conclude that red cells in the splenic vein are slightly more spheroidal than cells in the splenic artery(6).

Summary. 1. As pH of blood samples from 6 normal subjects was decreased, susceptibility of normal erythrocytes to mechanical trauma or their mechanical fragility was increased. 2. MF determinations on PNH and normal erythrocytes were done at various values of pH including the range for optimum complement activity in normal serum and it was found that mechanical trauma did not contribute to hemolysis of normal or PNH erythrocytes even though the PNH hemolytic mechanism was active. 3. It is postulated that increased red cell volume due to a shift of Cl^-, HCO_3^- and H_2O into the erythrocyte, may be the mchanism of increasing spheroidicity of the erythrocyte and the MF as a function of decreasing values of pH.

Conclusion. 1. MF of erythrocytes of the PNH patient in an active properdin system does not differ from MF of the same erythrocytes in an inactive properdin-complement system, nor does the MF of these erythrocytes differ from that of normal erythrocytes at values of pH between 8.0 and 5.9. 2. The hemolytic system of PNH, which requires properdin, complement and Mg^{++}, does not enhance susceptibility of PNH erythrocyte to hemolysis by mechanical trauma *in vitro*. Hemolysis of PNH and normal erythrocytes attributable to mechanical trauma increases progressively as pH of blood decreases until a critical point (pH 6.0-6.2) where MF rises rapidly.

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Metabolism of Radioactive Para-Aminobenzoic Acid in an *Escherichia coli* Mutant.* (22199)

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While metabolism of para-aminobenzoate (PAB) has been extensively investigated in the mammalian body(1,2), its function and biochemical transformations in microorganisms are still obscure. Conversion of PAB into folic acid and the citrovorum factor occurs in some organisms(3,4) although there is some question whether these are the active forms of PAB. A number of other routes of metabolism have been reported. Davis(5,6) has implicated PAB in the metabolism by *Escherichia coli* of tryptophane, tyrosine and other aromatic compounds, as well as vit. B_{12}. Ratner *et al.*(7) isolated a glutamic acid peptide of PAB from yeast, and Flynn *et al.*(8) have found in 2 strains of streptococci an antibiotic containing PAB and cytosine among its components. Sloane *et al.*(9) identified aniline and p-aminophenol as degradation products in *Mycobacterium smegmatis*. The present studies had their inception in the isolation by Lampen, Roepke, and Jones(10) of an X-ray mutant of *E. coli* which required exogenous PAB for growth but apparently did not utilize it in producing folic acid. Since the wild strain of this organism normally has no requirement for preformed PAB, it seemed of interest to investigate the fate of this compound labeled with C^{14} in such a mutant.

Materials and methods. PAB used was labeled in the carboxyl group with carbon 14 and had a specific activity of 0.037 mC/mg.[‡] The mutant of *E. coli* (ATCC No. 9723a) was grown on basal medium modified from that of Cohen and Arbogast(11) by use of 0.4% glucose. The medium of Lampen *et al.*(10) contained asparagine as well, but this substance was not necessary for maximum growth, and was omitted to facilitate isolations. Organisms were grown in 3 liter batches with aeration for 24 hours at PAB concentration of 0.01 μg/ml. Experiments in which PAB level was 0.05 μg/ml gave similar results. Bacterial suspensions were filtered on Buechner funnel using Celite as filter aid. The filtrate contained less than 20% of total radioactivity. The cells, plus filter-aid, were suspended in water and made up to 60% acetone-water (v/v) by addition of acetone. The residue was filtered off, re-extracted, and extracts, containing approximately half the total cell radioactivity, were combined. Subsequent extractions of cells with hot acetone, chloroform,

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[‡] Purchased from Atomic Energy Commission, Los Alamos Laboratories.

and petroleum ether removed practically no radioactivity. An additional 30-40% of the original total radioactivity of cells could be extracted with water. Extracts were lyophilized and kept at –20°C until they were analyzed.

TABLE I. R_f Values of Various PAB Derivatives.

Compound or extract	Solvent 1	Solvent 2
60% acetone extract	.15, .22, .48	.87–.95
PAB	.78	.69
Folic acid		.31, (.19)*
N^{10}-formyl folic acid	.36, .55, .75	.36, .55, .74 (.23, .55, .70)*
Natural citrovorum factor†	.55, .71	.46, .64
Leucovorin	.36, .55, .71	.49, .64, (.65)*
Pteroic acid		(.04)*
Pteroylglutamyl-alpha-glutamic acid		(.17)*
Pteroylglutamyl-gamma-glutamic acid		(.35)*
Pteroylglutamyl-gamma-glutamyl-gamma-glutamic acid		(.16, .34)*
p-aminobenzoylglutamic acid	.68	.90
p-aminobenzoylaspartic acid	.62	.88

* These figures from Wieland *et al.*(12).

† Obtained through the courtesy of Dr. K. Schwarz, N. I. H.

Results. Chromatographic separation of fractions. The bacterial extracts were subjected to ascending paper chromatography on Whatman No. 1 filter paper to investigate the nature of radioactive components. The behavior in the systems investigated led to the conclusion that PAB, folic acid, citrovorum factor, and several other related compounds were not among the bacterial metabolites, although good resolution of radioactive spots was never attained. The R_f values of various substances obtained in 2 systems, including results observed by Wieland *et al.*(12) are indicated in Table I. The solvent systems were 1) methanol, *iso*-butanol, water (1:1:1, v/v) and 2) 5% ammonium citrate buffer of pH 9 saturated with *iso*-amyl alcohol. Folic acid and its derivatives were located by ultraviolet absorption; PAB and its amino acid derivatives by the Bratton-Marshall aromatic amine reaction(13). Several of the folic acid compounds give more than one spot in certain solvents. Detection of the unknown was solely on the basis of direct radioactive assay of the paper strips, since there was insufficient material present for chemical identification or for spectrum analysis.

Because of the low recovery of radioactivity and poor definition of spots on the paper strips, column chromatographic separations were undertaken. Radioactivity was too tightly bound on Dowex 1 anion exchange resin, but chromatography on Dowex 50 cation exchange resin (10% cross-linkage, 200-400 mesh) provided separation. For elution 0.1 M ammonium acetate buffers were selected because they lent themselves to easy removal. The columns finally used were 100 cm by 0.9 cm, and were initially equilibrated with pH 3.32 buffer. Fractions of about 5 ml were taken and suitable aliquots were radioassayed. Three large radioactive peaks, denoted as B, C, and D in Fig. 1, were detected in the 60% acetone extract. The water extract contained mainly an additional peak, marked as fraction A, along with small amounts of B, C, and D. Fig. 1 shows typical elution patterns obtained at different pH values, PAB has never been seen in more than trace amounts and is eluted in pH 5.9 buffer. The supernatant medium was analyzed in the same chromatographic system and radioactivity was found to consist chiefly of PAB.

For comparison a series of pure compounds were subjected to the same separation procedure. Para-aminobenzoylglutamate and aspartate both emerge at the position of fraction C, but were definitely distinguishable from C

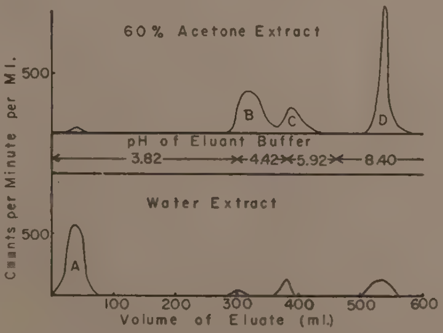

FIG. 1. Elution patterns of PAB metabolites.

by paper chromatography in a solvent of concentrated acetic acid, *iso*-propanol, concentrated ammonium hydroxide (3:2:1, v/v). Fraction C has an R_f of 0.30, while the 2 amides have R_f values of 0.78 and 0.74 respectively. Folic acid, formylfolic acid, and leucovorin are eluted in the general range of fraction D but again have been eliminated as possible metabolites by paper chromatography in the above solvent system, where D has an R_f of 0.30 while the 3 folic acid derivatives have values of 0.55, 0.80, and 0.64 respectively.

Acidic hydrolysates of all 4 metabolic fractions, prepared by refluxing with equal parts of concentrated hydrochloric and formic acids for 16 hours, and the corresponding basic hydrolysates, resulting from boiling with 1 M sodium hydroxide for 2 hours, were rechromatographed on the columns. In every case, PAB was identified as a major component of the hydrolysate.

It seems probable that the 4 metabolic fractions are closely related to each other, perhaps as degradation products, for on rechromatographing each separate fraction, some of the other fractions were formed. During this subsequent treatment fraction D was always formed in largest amount, and may represent the simplest compound.

The addition of methionine, asparagine or a mixture of other amino acids to the growth medium did not alter the elution pattern of the bacterial products, in spite of the observation of Lampen *et al.*(10) that some of these compounds spared a large portion of the PAB requirement of the mutant.

Growth of the mutant with PAB substitutes. A number of other compounds were tested for their ability to promote growth of the mutant. Growth was measured turbidimetrically at 430 mμ. It was felt that extension of such studies, initiated by Lampen *et al.* (10), might facilitate identification of the 4 fractions. Certain of these compounds have been implicated with PAB metabolism in other organisms, either by replacing its requirement for the compound or as metabolites. Some of these data are seen in Table II.

TABLE II. Growth Promotion of *E. coli* Mutant by Various Substances.

Drug		Conc. for half max growth—40 hr (μg/ml)
PAB		.001
p-aminosalicylic acid		.001*
p-aminobenzoylglutamic acid		.1
p-aminobenzoyl aspartic acid		.1
p-formamido benzoic acid		.1
p-formamido salicylic acid		.1
N-formyl-p-aminobenzoylglutamic acid		.1
N-formyl-p-aminobenzoylaspartic acid		.1
Folic acid		1.0
Formylfolic acid		1.0
p-aminohippuric acid		5.0
p-acetamidobenzoic acid		10
p-acetamidosalicylic acid		10
Pantothenic acid	No growth,	5
Aniline	*Idem*	5
Vit. B_{12}	"	5
p-hydroxybenzoic acid	"	5
3-hydroxy-4-aminobenzoic acid	"	5
p-aminophenol	"	5
N-acetyl-p-aminophenol	"	5
Pyridoxine	"	10
Leucovorin	"	1000

* Delayed.

In confirmation of Lampen *et al.*(10), the folic acid-like compounds were only weakly effective. Most other substances studied were without effect except for those in which PAB was coupled with an amino acid, or where the amino group was acylated. However since higher concentrations were necessary for comparable growth, it seems likely that these compounds become effective only after hydrolysis. The most active compound, other than PAB, was p-aminosalicylic acid. Delay in growth seen with this substance suggests that it is further metabolized before utilization, and indeed growth in the presence of p-aminosalicylic acid is inhibited by sulfadiazine as if it were PAB. Para-aminosalicylic acid, however, was not eluted from the columns at the position of any of the labeled fractions. The results of Lampen *et al.*(10) with regard to the PAB sparing action by amino acids and purines on growth of the mutant as well as inhibition by sulfadiazine were confirmed. The latter substance inhibits completely at a concentration of 1 μg/ml in the presence of 0.0005 μg/ml of PAB whereas 4 other metabolic inhibitors, A-methopterin, desoxypyridoxine, 8-azaguanine and 6-mercaptopurine, were without effect at 10 times this level. The fact that A-methopterin did not inhibit growth

TABLE III. Relative Growth Promotion by PAB Metabolites for 3 Microorganisms.

Compound or metabolite	mμg/ml of medium for one-half maximal growth		
	E. coli (ATCC 9723a)	*S. lactis* R. (ATCC 8043)	*L. citrovorum* (ATCC 8081)
PAB	1	* (100)	* (100)
Folic acid	1000	.2	* (10)
Leucovorin	* (1000)	.2	.1
Fraction A†	3	* (65)	* (140)
B†	* (50)	* (100)	* (200)
C†	* (50)	16	36
D†	5	4	8

* No growth observed at highest concentration tested; concentration given in parentheses.
† Weights of PAB computed from radioactive assay and converted to equivalents of folic acid or leucovorin.

of mutant indicated that folic derivatives might not be closely involved in the anabolic metabolism.

Bacterial growth studies with metabolic fractions. Ability of each of the 4 fractions to support growth of the mutant was then investigated. The possible similarity to folic acid and citrovorum factor was also studied by the ability to promote multiplication of *Streptococcus lactis* and *Leuconostoc citrovorum,* using commercial Difco media(14) for these microbiological assays. Suitable controls were run with mixtures of the appropriate growth factors and the 4 fractions, in order to demonstrate that none of the 4 fractions contained a growth inhibitor. Table III gives the relative concentrations of each fraction for growth of these 3 organisms.

It was possible that the growth stimulation seen in Table III by certain fractions might have resulted from the presence of unlabeled folic acid or leucovorin present as a contaminant. Therefore paper chromatograms of all 4 fractions were prepared under aseptic conditions in 2 solvent systems and the dried sheets were placed on agar plates seeded with *S. lactis* or *L. citrovorum* but devoid of the limiting growth factors. Growth of the organisms was seen only with fractions C and D and was localized at the site of radioactivity. Contaminating folic acid or leucovorin were apparently absent from the fractions, because no growth could be seen where these substances would have been located on the paper strip.

Discussion. While insufficient material has prevented chemical identification of the metabolites of PAB in this mutant of *E. coli,* the evidence suggests considerable similarity exists between certain of the metabolites and the citrovorum factor. It is of interest that Nichol *et al.*(15) recently reported synthesis from folic acid of various labile precursors and conjugates of citrovorum factor by pigeon liver and *S. faecalis.* Failure to stimulate growth of the mutant by the fractions at concentrations quite comparable to those at which PAB is active might suggest that all fractions are derived from some highly active metabolite degraded as a result of the isolation procedure or that one or more of the fractions consists of a mixture of active and inactive metabolites. The evidence suggests that the substances are quite labile. It remains for further purification and isolation of much larger amounts to elucidate their structure and possible function.

Summary. Radioactive p-aminobenzoic acid has been incubated with an x-ray mutant of *Escherichia coli* which requires this substance as a growth factor. By column chromatography 4 soluble radioactive fractions have been isolated from the cell bodies of the organism, none of them consisting of the original substrate. Two of the fractions possess citrovorum factor activity, but none of them are identical with folic acid, citrovorum factor or certain of its derivatives.

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Effects of Guinea Pig Serum on 6C3HED and AKRL1 Lymphoma Cells Implanted in Various Hosts.* (22200)

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Cells of Lymphoma 6C3HED promptly begin to die when serum from normal guinea pigs is injected into C3H mice in which they are growing(1); yet the lymphoma cells remain viable when they are held in contact with guinea pig serum during several hours at 37°C *in vitro*(1,2). The facts suggest that the animal host somehow participates in the process whereby these lymphoma cells are killed *in vivo*, perhaps by supplying special conditions essential for the process, or by furnishing some substance—an isoantibody, for example, or one or another of the components of complement—that might work with guinea pig serum in producing the effect(1,2). A number of questions related to the facts just mentioned are raised by a more recent observation—namely that cells of Lymphoma AKRL1 continue to proliferate without restraint when normal guinea pig serum is given to AKR mice carrying them(3). Do the AKR mice fail to supply such conditions as might be required for the effectiveness of guinea pig serum *in vivo*, or are they devoid of some essential host factor? Is there an intrinsic difference in cells of Lymphoma 6C3HED and those of Lymphoma AKRL1, the former being susceptible to the effects of guinea pig serum *in vivo* while the latter are insusceptible? The findings of several experiments, now to be given, bear on these questions.

Results. The experiment of Fig. 1 shows that the cells of Lymphoma 6C3HED were inhibited quite conspicuously when guinea pig serum was given intraperitoneally to AKR mice into which 10 million lymphoma cells had been implanted in each of 2 subcutaneous sites by a technic already described(1), and the finding was confirmed in a subsequent experiment done in precisely the same way. Furthermore, in the experiment of Fig. 1 the inhibitory effect of guinea pig serum was even more marked in the alien and resistant AKR hosts than in the C3H hosts of the sort in which this lymphoma had originated (and which had been implanted with 1 million 6C3HED lymphoma cells in each of 2 subcutaneous situations). This result suggests that the effects of guinea pig serum might depend upon, or be enhanced by, some resistance manifested by the animal

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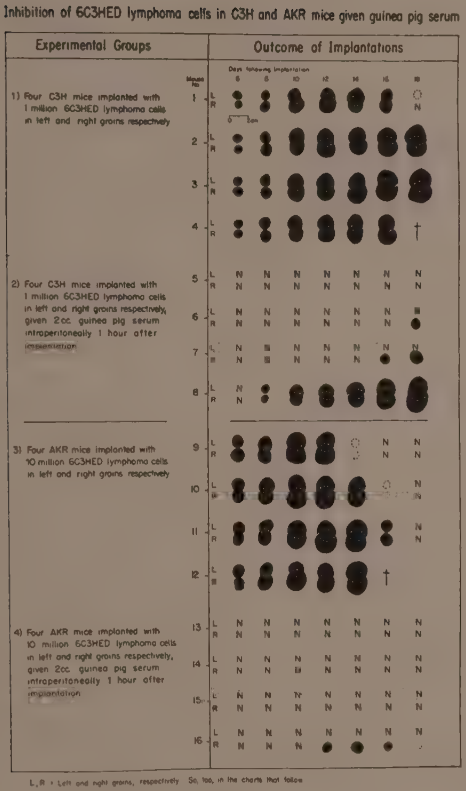

FIG. 1.

hosts, this being the greater in the mice of alien breed. More will be said about this further on.

Next the converse experiment was done. Ten million cells of Lymphoma AKRL1 were implanted in each groin of 8 C3H mice (which were presumably resistant to them since the cells had originated in AKR mice), and 1 million cells were implanted in each groin of 8 AKR mice of the sort in which the lymphoma had originated(3). Four mice of each breed were left untreated as controls and the others were given, approximately 1 hour after implantation, 2 cc of normal guinea pig serum from the same pool as that used in the experiment of Fig. 1. The guinea pig serum had no inhibitory effect whatever on subsequent growth of lymphoma cells in either native or alien hosts; for the cells implanted in treated and untreated AKR mice grew and formed palpable tumors of approximately equal size, and these all enlarged until death of the animals, as careful chartings showed. Furthermore, the AKRL1 cells that had been implanted in the alien C3H hosts regularly grew to form tumors 10-16 mm across on the 10th day following implantation; but these tumors dwindled and disappeared in 5 of the 8 mice between the 10th and 19th days, while growing progressively in one of the untreated and 2 of the treated animals. The process of regression, although essentially similar in treated and untreated animals, was somewhat more abrupt in the untreated animals, the finding suggesting either that the guinea pig serum might have interfered to some extent with the process of regression or that it might have enhanced growth of these lymphoma cells under these conditions.

In a further test of this sort 2 additional lymphomas of AKR mice — AKR13 and AKR17(3)—were used. Ten million cells of each type were implanted in both groins of 8 C3H mice, 4 of which were left untreated as controls while the others were given 2 cc of guinea pig serum intraperitoneally one hour after the implantations. During the next 6-10 days, tumors measuring up to 17 mm across appeared at every implantation site, the growths in treated animals being precisely like those in untreated controls; within the following 10 days, however, the growths all dwindled and disappeared (Fig. 2), this process too being essentially the same in the treated and untreated groups. In concurrent tests, the guinea pig serum did not influence the outcome of implantations of these cells in AKR mice, the findings confirming observations already published(3).

F1 hybrid C3H x AKR mice were employed in the several further experiments. In the most definitive of these, 500000 6C3HED lymphoma cells were implanted in one groin of 8 hybrid mice, and 500000 AKRL1 lymphoma cells were implanted in the opposite groin; half of the implanted mice were then kept as controls and the others were given normal guinea pig serum intraperitoneally, 2 cc

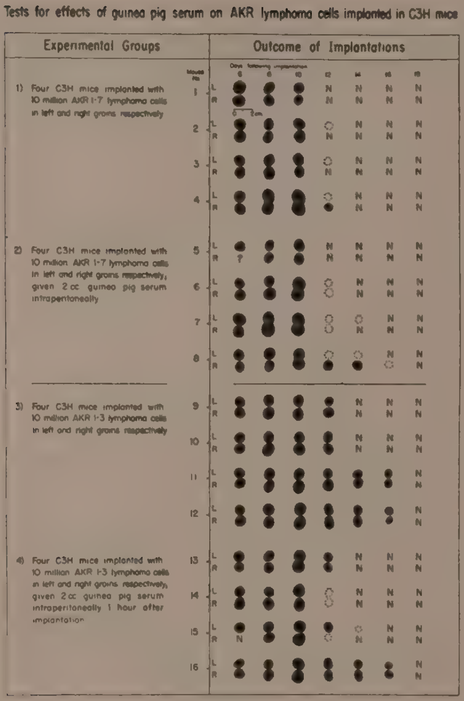

FIG. 2.

approximately 1 hour after the implantations had been made and 2 cc on each of the following 2 days. Fig. 3 shows the outcome. In the untreated control mice, tumors 10 to 14 mm across were palpable in both groins on the 8th day following the implantations, and these enlarged progressively until death of the hosts between the 18th and 19th days. In the 4 treated mice, tumors similar to those of the control animals appeared in the groins which had been implanted with AKRL1 lymphoma cells, and these growths also enlarged progressively; but the sites implanted with 6C3HED cells remained devoid of tumors. In a further experiment 500000 6C3HED cells were implanted in both groins of 8 hybrid mice and the same number of AKRL1 cells were implanted in each groin of 8 additional animals; half of the mice of each implantation were then given 3 injections of guinea pig serum as in the preceding test. The 6C3HED cells grew as before in the untreated animals, but failed to form palpable tumors in those given guinea pig serum; the AKRL1 cells formed tumors in the treated and untreated hosts which appeared promptly and grew rapidly and progressively in all instances.

Mention was made above of the possibility that the effects of guinea pig serum *in vivo* might depend upon or be enhanced by some resistance provided by the animal hosts and directed against the lymphoma cells as foreign tissue. If this should prove to be the case, then the effects should be more marked against lymphoma cells that had grown for some days in alien hosts; for under these circumstances such immune responses as the hosts were capable of making should have been stimulated. An experiment was done next to test the possibility. Ten million cells of each of 3 AKR lymphomas previously employed—AKRL1, AKR13, and AKR17—were implanted in both groins of 8 (alien) C3H mice. Nothing further was done to any of the animals until 6 days later, at which time palpable tumors measuring approximately 1 cm across were present at all implanted sites. On the 6th, 7th, and 8th days, 2 cc of normal guinea pig serum were given to 4 of the mice that

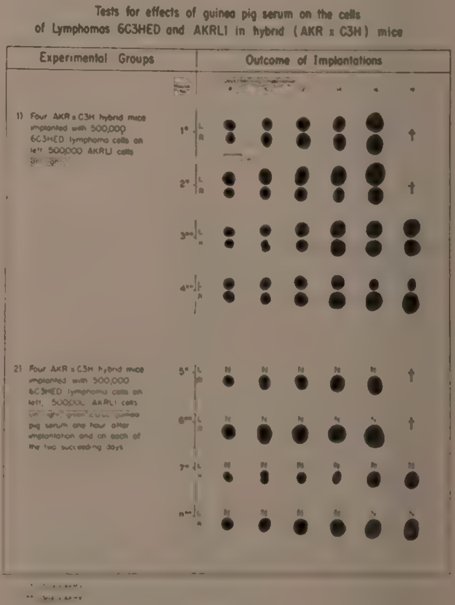

FIG. 3.

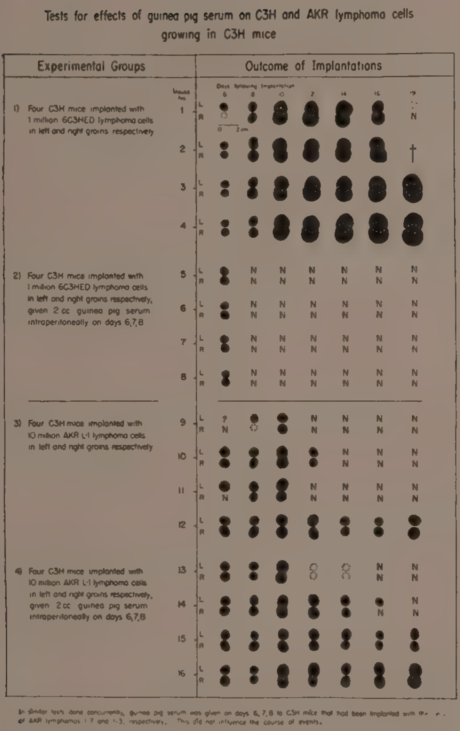

FIG. 4.

had been implanted with each type of lymphoma. The outcome in the mice implanted with 6C3HED and AKRL1 cells is shown in Fig. 4. It can be seen that the injected guinea pig serum promptly brought about regression of the 6C3HED lymphomas but had no such effect on the AKRL1 growths. The guinea pig serum given in this way to the C3H mice carrying Lymphomas AKR13 and AKR17 was likewise devoid of effect, though to conserve space the findings are not given in the chart.

The findings just described were extended in several further experiments. In the first of these 6 million cells of lymphoma 6C3HED were implanted in 6 mice of each of 2 alien strains heretofore unused (strains A and DBA), and in 6 C3H mice also; half of the animals were kept untreated as controls while the others received 2 cc of guinea pig serum on the day of implantation and on each of the next 2 days as well. Tumors appeared promptly in the untreated mice of all 3 breeds and grew progressively to bring about death of the hosts between days 11 and 18, except in one mouse of strain A in which the growths regressed between the 10th and 12th days. Palpable tumors did not appear in any of the mice given guinea pig serum. In a similar experiment, 6 million AKRL1 cells were implanted in both groins of 6 strain A and 6 DBA mice, half of the animals of each breed being treated with guinea pig serum as before. In the strain A mice, palpable tumors 9 to 11 mm across appeared in all treated and untreated animals 7 to 8 days following implantation; the growths in both groups of mice enlarged during the ensuing 4-6 days but thereafter dwindled and disappeared, the process of regression again being essentially the same in treated and untreated animals. In all 3 untreated DBA mice and in one of those given guinea pig serum the AKRL1 cells failed to form palpable tumors, but in 2 of the treated animals they gave rise to bilateral growths that appeared between 7th and 11th days and enlarged progressively until the 20th day when the animals were discarded. A further indication that guinea pig serum can enhance growth of AKRL1 cells *in vivo* was provided by an additional experiment. Eight C57 mice were implanted with 1.5 million AKRL1 lymphoma cells and half of the animals were then given 2 cc of guinea pig serum. Again, the untreated animals failed to develop palpable tumors, but all 3 of those given guinea pig serum developed growths, which measured 12-14 mm across on the 7th day following implantation, enlarged during the ensuing 2 days, and thereafter abruptly disappeared. It remains to be seen whether these findings have a relation to the observations of Kaliss which indicate that certain antisera may enhance growth in alien hosts of transplanted cancers of other types (4,5).

Summary. In the experiments here given, serum from normal guinea pigs acted regularly and powerfully against cells of Lymphoma 6C3HED when these were implanted in mice of various breeds (C3H, AKR, A, DBA, and hybrid C3H x AKR). But it did not inhibit the cells of Lymphoma AKRL1 in hosts of the above mentioned sorts, and it likewise failed to inhibit growth of cells of 2 additional lymphomas (AKR13 and AKR17) in C3H mice. The findings make it plain that guinea pig serum does not act against the 3 types of AKR lymphoma cells even when these are implanted in alien hosts; indeed in some of the experiments guinea pig serum seemed to enhance the growth of AKRL1 cells in alien hosts. Considered together the observations weigh heavily against the supposition that the mouse host provides natural or induced isoantibodies which act in conjunction with guinea pig serum in killing the cells of Lymphoma 6C3HED *in vivo*, and they support the inference that cells of Lymphoma 6C3HED differ intrinsically from those of Lymphoma AKRL1, the former being susceptible to the effects of guinea pig serum *in vivo* while the latter are insusceptible.

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Effect of Tumors on Level of Plasma Amino Acids of Eviscerate Rats.* (22201)

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It was expected that the presence of a growing tumor would suppress the rise in plasma amino acids which occurs following evisceration. The contrary is true. The rise is more rapid in eviscerate rats which are hosts to a Walker carcinoma.

Methods. Male rats of the Sprague-Dawley strain were fed Rockland rat diet. Suspensions of tumor were implanted intramuscularly into each thigh near the trunk and permitted to grow for periods of 4 to 14 days. At a weight of approximately 250 g non-fasted rats were anesthetized with cyclopal sodium and functionally eviscerated. Some of the animals were kept without infusions and others were given continuous intravenous infusions of glucose with and without insulin for periods of either 3 or 6 hours. Heparinized blood was collected by cannula from the abdominal aorta and the level of plasma amino acids determined by the method of Hamilton and Van Slyke(1).

Results. The conditions of individual experiments and the results are shown in Table I. The values are grouped according to the combined weights of tumors of each rat at autopsy. The level of plasma amino acids is not elevated in non-eviscerated tumor hosts. The average values for plasma amino acids increased more rapidly in tumor-bearing rats, the extent of rise showing a positive relationship to the weight of the tumors. Although insulin suppresses the rise in plasma amino acids in all eviscerate animals(2) it does not prevent a more rapid rise in tumor hosts. Whether the extra plasma amino acids arise from the tumor itself, or from normal tissues which are catabolized more rapidly in the presence of a tumor, is not known to us. We have no information about changes in tumor metabolism following evisceration.

Summary. Following evisceration the level of plasma amino acids rises more rapidly in rats which are hosts to a Walker carcinoma

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* This work was supported by grants from the American Cancer Society as recommended by the Committee on Growth and from the United States Public Health Service.

TABLE I. Level of Plasma Amino Acids in Rats Bearing Tumors. Averages and standard errors.

Glucose load, mg/100 g/rat/hr	Insulin, units/24 hr	Hr	No. rats	Tumor wt, g	Plasma amino acids, mg %
0	0	0	12	36.94 ± 5.46	3.97 ± .28
0	0	3	12	0.0	9.35 ± .33
0	0	3	12	2.34 ± .39	11.55 ± .45
0	0	3	8	8.98 ± 1.12	14.26 ± .88
0	0	3	10	22.63 ± .79	15.09 ± .79
0	0	3	10	37.72 ± 2.73	15.83 ± .60
0	0	6	8	0.0	15.00 ± .82
0	0	6	8	1.68 ± .27	17.91 ± 1.13
0	0	6	6	8.15 ± 1.36	20.77 ± .76
0	0	6	6	25.36 ± 1.50	20.26 ± 1.13
0	0	6	10	53.78 ± 6.21	21.64 ± 1.11
70	0	3	17	0.0	8.82 ± .09
70	0	3	17	19.1 ± 5.46	10.59 ± .25
70	16	3	17	0.0	4.48 ± .11
70	16	3	17	19.76 ± 3.41	7.30 ± .44

than in similar rats without tumors. The extent of rise shows a positive relationship to the size of the tumors at autopsy. The differences are evident in the presence and absence of insulin.

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Studies on Agammaglobulinemia VI. Hemostasis in Patients with Agammaglobulinemia.* (22202)

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The discovery of agammaglobulinemia(1) has opened a new field of investigation in immunology and protein metabolism. Appreciable information concerning these patients has accumulated, especially in the field of immunology(2). In all conditions with a congenital or acquired defect of protein synthesis the problem arises whether one is dealing with an isolated error of metabolism or with a group of deficiencies. Typical examples of isolated defects of protein synthesis are the congenital disturbances of coagulation: *e.g.* afibrinogenemia, the various subgroups of hemophilia, hypoprothrombinemia, etc. Examples of combined defects of protein synthesis are found with severe liver damage, where hypoprothrombinemia is usually associated with deficiencies of prothrombin conversion factors, hypofibrinogenemia and hypoalbuminemia. The association of a number of defects can usually be traced to a dysfunction of the common organ of synthesis.

Because of the existing controversy about the site of synthesis of gamma globulins, it was considered of interest to determine the concentration of the presently known clotting factors and wherever possible to evaluate the adjustment of these factors in response to stressful situations in patients with demonstrated inability to synthesize gamma globulin. Complete tests of hemostasis were performed in 4 children with congenital agamma-

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* This study was aided by U. S. Public Health Service Grants, Helen Hay Whitney Foundation, American Heart Assn. and the Minn. Heart Assn.

† American Legion Memorial Heart Research Professor of Pediatrics.

globulinemia and 3 adults in whom the agammaglobulinemia was probably acquired (3). With one exception (case L.L.) all patients were males. During the course of study, one adult developed homologous serum hepatitis and expired in hepatic coma. The changes in clotting factors as the liver failure progressed will be reported below. In 2 children the fibrinogen concentration changed during a febrile episode. Otherwise all tests were carried out during a state of clinical well-being in all patients.

Material and methods. Previously described methods have been applied for the *collection and processing of blood, plasma and serum*(4), the determination of *recalcification time*(4), *thrombin time*(4), one-stage *prothrombin consumption*(4), *prothrombin concentration*(5), *labile and stable prothrombin conversion factors*(6) and *fibrinolysis*(6). *Bleeding time*—Method of Ivy(7). *Coagulation time of venous blood*—Modified 3 tube Lee-White method. *Prothrombin time*—Method of Quick(8). *Platelet count*—Method of Rees-Ecker(9). *Capillary fragility*—Rumpel-Leed test(10). *Fibrinogen determination*—Method of Ratnoff(11).

Results. The tests of hemostasis presented in Table I show no deviations from normality except for a high platelet count in all children and in one adult. Of these tests only the determination of fibrinogen, platelets and prothrombin concentration are specific and quantitative. The activity of other clotting factors can only be determined by inference from a group of additional tests. The normal consumption of prothrombin indicates that the various plasma precursors of thromboplastin (Anti-Hemophilic Globulin, Plasma Thromboplastin Component, Plasma Thromboplastin Antecedent, fourth plasma thromboplastin component of Spaet *et al.*(12) and the factor deficient in Hageman's trait(13)) must be present in normal concentration. The determination of prothrombin with the modified 2-stage method(5) revealed a normal rate of prothrombin conversion in all patients. This proves that both the labile and the stable prothrombin conversion factors are present in normal amounts in the plasma of agamma-

TABLE I. Tests of Hemostasis in 7 Patients with Agammaglobulinemia.

	Congenital group				Acquired group			
	W.A., 6 yr	T.A., 1 yr	E.S., 7 yr	J.S., 7 mo	F.H., 58 yr	G.T., 29 yr	L.L., 30 yr	Normal
Bleeding time, min.	3	2	2	2	3	1	5	max 6
Clotting time, min.	20	23	20	23	20	21	19	max 30
Prothrombin time, sec.	12.5	12.6	12.4	13.8	12.4	14.0	13.4	12.0–14.0
Recalcification time, sec.	150	90	150	120	140	150	140	max 180
Platelets, per mm^3	558 × 1000	594 × 1000	390 × 1000	468 × 1000	256 × 1000	194 × 1000	189 × 1000	150–220 × 1000
Clot retraction	normal	normal	normal	normal	normal	normal	normal	
Rumpel-Leede test	neg.	neg.	neg.	neg.	neg.	neg.	neg.	
Fibrinogen, mg/100 ml	320	322	253	291	262	364	320	250–350
Prothrombin conc., U/100 ml	305	270	270	270	305	315	270	260–360
Prothrombin consumption								
Serum prothrombin time, sec. (2 hr after venipunct.)	43.4	35.7	39.0	56.0	30.6	28.4	45.6	above 25.0
Thrombin time, sec.	15.5	14.0	14.6	14.0	14.0	16.0	15.6	14.0–16.0
Fibrinolysis	absent	absent	absent	absent	absent	absent	absent	

globulinemic patients. Finally the normal thrombin time excludes any disturbance in the last phase of normal coagulation, *i.e.* in the conversion of fibrinogen to fibrin.

The changes in clotting factors in case F.H. during the course of a fatal hepatitis were in no way different from the changes observed in progressive hepatic failure in a normal individual: the drop in prothrombin concentration from 305 to 66 units/100 ml preceded the fall of fibrinogen from 262 to 110 mg/100 ml and the terminal thrombocytopenia of 72,000 per cumm. The temporary rise of fibrinogen to 449 mg/100 ml in patient J.S. during the course of a febrile episode after the administration of typhoid vaccine is also characteristic of the response observed in normals. Similarly a rise of fibrinogen from 210 to 428 mg/100 ml occurred in patient E.S. during an attack of pneumococcal pneumonia. Upon recovery the fibrinogen level fell to a normal level of 240 mg/100 ml. At no time during the course of these episodes was it possible to detect any gamma globulin in the serum of either patient by electrophoresis. It appears, therefore, that the changes in coagulation factors observed in certain disease states occur in agammaglobulinemia in identical fashion as in normal individuals.

Discussion. The elevation of the platelet count in all 4 cases of congenital agammaglobulinemia and in one adult, who probably had an acquired type of agammaglobulinemia, points toward some functional aberration in the bone marrow. It is of interest in this connection that agammaglobulinemia is characterized by other abnormalities in the hematopoietic system. Among these is the virtual absence from the lymph nodes, spleen and bone marrow, of plasma cells in all cases thus far studied(14). In certain instances absence of eosinophils from the peripheral blood and bone marrow, neutropenia and lymphopenia(3) have reflected the disturbed function of the reticuloendothelial system.

The normal concentration of the plasma clotting factors has several fundamental implications. (1) It suggests that none of the protein factors involved in the coagulation of blood is a gamma globulin. (2) It indicates that synthesis of all the coagulation moieties produced by the liver is intact in patients with agammaglobulinemia. The liver is the source of fibrinogen, plasma thromboplastin component, prothrombin and the stable and labile prothrombin conversion factors. (3) It supports the concept that the metabolic defect underlying agammaglobulinemia is an isolated deficiency of a single enzyme system. Observations thus far reported(2), including the data submitted here, indicate that synthesis of all proteins, save gamma globulin and the closely related beta 2 globulin is normal in these patients. It also seems of significance that changes in clotting factors during disease states occurred in normal fashion. All these findings are in keeping with previously published data(3) which showed no evidence of abnormality of hepatic function in patients with agammaglobulinemia. These data permit the conclusion that synthesis of gamma globulin and antibody is not directly associated with synthesis of the protein factors involved in hemostasis and support the concept that gamma globulin and antibody are not formed in the parenchymal cells of the liver.

Summary and conclusions. 1. The activity or concentration of the clotting factors recognized at the present time have been determined in 7 patients with agammaglobulinemia. Four of the patients had the congenital-hereditary form of agammaglobulinemia and three had the acquired form of the disease. 2. An elevated platelet count was observed in 5 of the 7 patients. No abnormalities in plasma clotting factors were found. 3. Data presented are consistent with the following conclusions: (a) none of the plasma coagulation factors is a gamma globulin, (b) agammaglobulinemia is probably the result of an isolated deficiency in protein synthesis, (c) the deficiency of gamma globulin synthesis in these patients does not involve the liver but rather depends on an anomaly of protein metabolism existing elsewhere in the reticuloendothelial system.

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Water Intake of Guinea Pigs as Parameter in Oral Drug Therapy of Experimental Irradiation.*† (22203)

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In the study of the effects of drugs on X-ray-induced mortality of experimental animals, the use of peroral route is preferable in many instances to injection because of increased bleeding tendency exhibited by some laboratory animals. This is particularly true with respect to guinea pigs. In this species the pronounced bleeding tendency may cause fatal bleeding at the site of injection. Oral drug administration is usually performed by tube feeding, to control amount of medication as well as possible. This procedure is, however, not suitable for irradiated animals since these are particularly sensitive to infections. The small lacerations caused by such forced feeding experiments may open ports of entry for bacteria and thus superimpose a death rate caused by infection, which would becloud true results of such studies.

Administration of water-soluble drugs with the drinking water appeared, therefore, as a method of choice. Since, obviously, the amount of drug given in this manner would depend on water consumption of experimental animals, it was necessary to obtain information about their drinking habits.

Methods. Fifty-one male guinea pigs of the Institute stock with an average weight of 450 g (range 250-660 g), were kept in individual cages in air-conditioned quarters. Preliminary investigations had indicated that guinea pigs kept on our standard diet B, consisting of guinea pig pellets, lettuce, and a half orange daily, were drinking quite irregularly. To force experimental animals to drink, lettuce and oranges were withdrawn from the diet. Instead they were offered 235 cc of water to which was added 50 mg vit. C. The water was

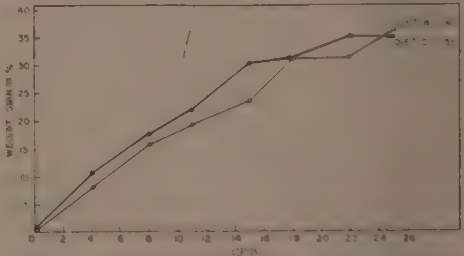

FIG. 1. Comparison between wt gains of guinea pigs kept on standard diet B and experimental diet C (dry food and drinking water plus 50 mg Vit C/235 cc). Figures in parentheses in this and subsequent curves designate No. of animals.

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* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† The technical assistance of W. A. Sterling HMC, USN, R. L. Devault, HM1, USN, and R. H. Crutcher, Biological Aid, is acknowledged with thanks.

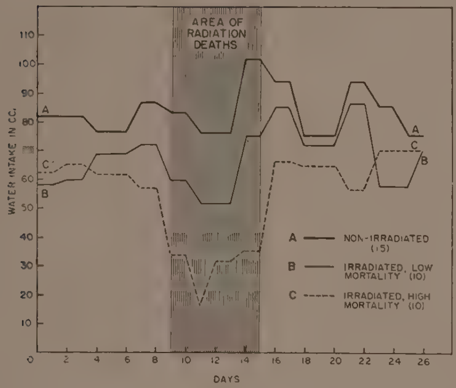

FIG. 2. Analysis of influence of X-ray-induced mortality on daily water intake of guinea pigs. Note decreased water intake in irradiated animals between 9th and 15th postirradiation day is proportional to percentage mortality caused by respective X-ray doses.

given in bottles which were coated with aluminum paint to preclude deterioration of the vitamin in daylight. The amount of water consumed was measured every other or third day.

Results. The first step was to ascertain whether this diet was satisfactory. The weight gain of 16 guinea pigs kept on the usual laboratory diet B was compared with that of 15 animals kept on the new diet C. The result is shown in Fig. 1 which indicates that there was no important difference in weight gain between these 2 groups.

The 15 guinea pigs kept on diet C for 26 days drank an average of 83.6 cc (stand. dev. ± 14 cc). A similar study was then performed on 20 irradiated animals, 10 of which had been exposed to the LD 30/26 days of 2000 KV X-rays and 10 animals which had received LD 80/26 days of 200 KV X-rays. The water consumption in this group averaged 61.7 cc (stand. dev. ± 14.8 cc). Fig. 2 shows in detail the variation in water intake during 26 days' observation. Curve A represents water consumption of non-irradiated animals, curve B that of animals exposed to X-ray dose causing a low mortality, and curve C that of animals exposed to the dose causing a high mortality rate. The time during which death occurred in irradiated animals is marked. There was no important difference in water consumption between groups B and C until the 9th postirradiation day. At this time some of animals showed symptoms of radiation illness and deaths began to occur in both groups. However, with occurrence of the first fatalities, a notable reduction in water intake in both groups took place, more pronounced in the group exposed to the X-ray dose causing the higher mortality. This observation induced us to consider water intake of survivors separately. In Fig. 3, curve A represents water consumption of non-irradiated animals, curve B shows that of the total group of irradiated animals, and curve C that of all survivors. As this graph indicates, those animals which were to survive, continued to consume water at a fairly constant rate. It is thus clearly recognized that reduction in water consumption was due to the fact that the animals which died reduced water intake shortly before expiration. Some stopped drinking 24 hours before death occurred.

Discussion. Water consumption of irradiated animals during the first week after irradiation was reasonably uniform irrespective of radiation dose. This seems to permit administration of a standard drug dose by mouth with a satisfactory amount of accuracy during this period which is most important for postirradiation protection. The standard procedure of this laboratory, for the evaluation of drugs on lethal effect of total-body

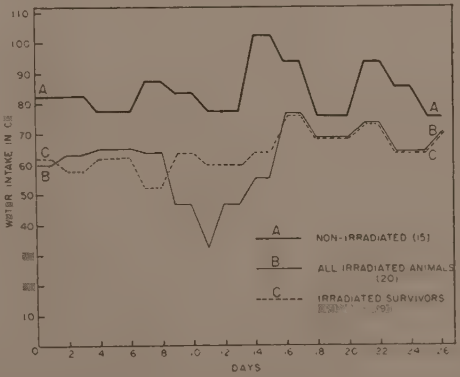

FIG. 3. Comparison of water intake of irradiated animals surviving X-ray irradiation (Curve C) with that of all irradiated animals (Curve B). Note that Curve B does not show the drop in water intake.

radiation, is to administer one fixed drug dose for 5 days after irradiation without adjusting medication to changes in weight of individual animals. The procedure of oral drug administration as outlined appears just as accurate as the injection method.

The oral route offers the advantage of administering larger total drug doses to animals by extending time of medication and of accomplishing this without additional stress to the animals. Extension of injections over equally long periods of time raises the obstacle of burdening the irradiated animal with larger amounts of vehicle substances which may in themselves adversely affect survival rate, as shown by us(1) and others(2) with respect to saline. The saving in time otherwise needed for injections is a further advantage of oral drug administration.

Summary. 1. Drinking habits of guinea pigs kept on a diet of dry food and drinking water have been studied to ascertain the amount of daily water consumption which may serve as a parameter for oral drug administration. 2. There is a difference between water intake of non-irradiated and irradiated animals. 3. This difference is chiefly accounted for by reduced water intake of animals succumbing to irradiation, which stopped drinking prior to death. 4. Water consumption of irradiated animals is not materially altered during the critical first 8 days after irradiation, irrespective of death rate produced later on. 5. Use of oral administration of water-soluble drugs with drinking water appears therefore possible with a sufficient degree of accuracy.

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A Urinofecal Separator for Laboratory Rat. (22204)

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The problem of obtaining urinary and fecal samples free from intercontamination is a major obstacle to some studies of absorption and metabolism utilizing laboratory animals. In the course of a study of the efficiency of absorption of dietary thiamin by the rat, using a conventional apparatus, serious errors attributable to contamination of fecal samples with urine were encountered. Subsequently a glass separator of new design was constructed which has been shown to effect this separation with a high order of efficiency. The design and operation of this apparatus are described herewith.

The construction of the separator is outlined in Fig. 1. An essential feature is the flared funnel stem which serves to draw the urine away from the pathway of the feces, which fall directly into the feces tube. Glass-to-glass contact must be maintained between tip of funnel stem and the inside surface of the separator, so that urine may flow readily around the interior of the bulb and be drained off by the urine tube. Urine is prevented from falling directly into the feces tube by the glass cone which rests on three legs ½ in. long. The feces tube is flared to 35 mm O.D. at the top, thereby being extended beneath the shoulder of separator bulb. The wire cage was constructed according to a design obtained from the Nutrition Division, Food and Container Institute, Quartermaster Corps, Chicago, Ill.

The whole apparatus may be attached to a ring stand by means of a 6 in. iron ring to support weight of the funnel and cage, and by 2 clamps fastened to neck of separator and to the feces tube, respectively.

The efficiency of the separator was tested according to the following scheme. Two nor-

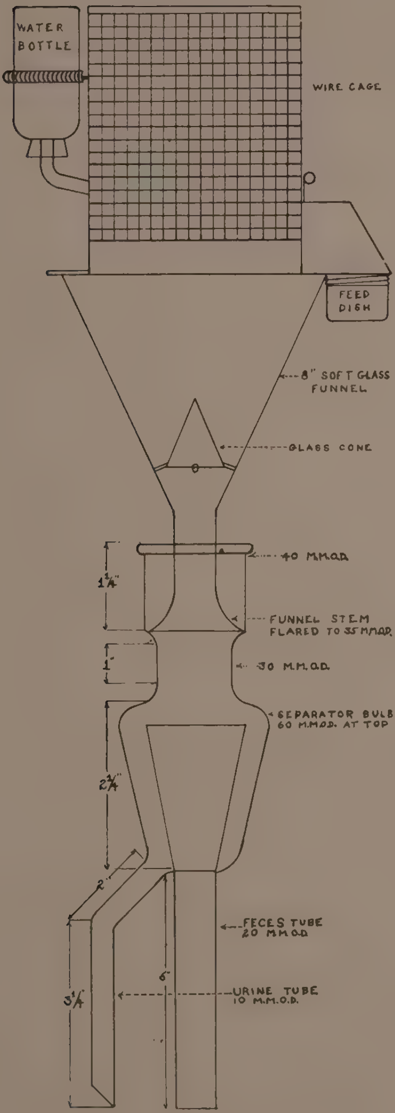

FIG. 1.

mal rats, A and B, weighing about 100 g, were placed in the wire cage. Rat A, which had been fasted during the previous 24-hr period, was given an intraperitoneal injection of 120 μg of radioactive thiamin (thiazole-2-C^{14}) having a specific activity of 6 μc/mg. Rat B had been fed a complete diet *ad libitum* during the preliminary period. A collection period of 24 hr ensued, during which time no food was offered. Throughout this interval a composite urine sample from both rats was collected, while the feces sample was derived predominantly from Rat B. Feces excreted by rat B were identified by means of a ferric oxide marker and any pellets excreted by rat A were discarded. At end of collection period the feces from rat B were hydrolyzed using papain and clarase, filtered, and the radioactivity of filtrate was measured by plating, using a Tracerlab windowless flow counter and a Nuclear scaler. Radioactivity of total urine was also determined and efficiency of the system was calculated as percent of urinary radioactivity which appeared in the feces of rat B.

Based upon 12 such tests, the average radioactivity in the feces, expressed as a fraction of total urinary radioactivity, was 0.11% with a standard deviation of 0.08%. No contamination of urine by feces was evident from visual examination.

The performance achieved by the apparatus described is sufficient for most research purposes. By appropriate modifications in scale, the separator may be adapted to smaller or larger laboratory animals than the growing rat. By introducing ground glass fittings and a glass cage, the apparatus also has been converted to a closed system for the simultaneous collection of respiratory gases. Some increase in ease of handling and in rigidity has been attained by the use of a pyrex funnel with the stem fused directly to the neck of the separator.

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ERRATUM

Oct. 1955, Article 21941, by Greenway and Quastel, Column heading, Table I should read "μl CO_2 above endogenous evolved in 1 hr".
P. 73, col. 2, line 33 "0.25 *M*" should read "0.025 *M*."

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SECTION MEETINGS

BALTIMORE	
University of Maryland Hospital	January 20, 1956
CLEVELAND, O.	
Western Reserve University	December 19, 1955 January 16, 1956
DISTRICT OF COLUMBIA	
George Washington University	February 2, 1956
ILLINOIS	
Veterans Admin. Hospital, Chicago	January 17, 1956
MINNESOTA	
University of Minnesota	January 25, 1956
NEW YORK	
New York Academy of Medicine	January 11, 1956

Vesicular Stomatitis Virus in Tissue Cultures and Cell Suspensions. (22205)

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The utilization of cultured tissues and cells for the production and assay of viruses has achieved widespread application following the epochal work of Enders, Weller and Robbins (1). The direct use of suspensions of tissue fragments and cells for virus propagation has had, on the other hand, much less appeal even though Frenkel and van Waveren proved the practicability of the method with vaccinia virus nearly 20 years ago(2). More recently, Frenkel employed this method in the production of foot-and-mouth disease virus for use in commercial vaccines(3). A practical test for the determination of virus concentration directly in cell suspensions has been lacking until recently and this appears to have been responsible, in part, for the heretofore limited use of such suspensions in virus studies. Salk *et al.*(4), Lipton and Steigman(5) and Robertson *et al.*(6) have now shown that cell suspensions can be used for the assay of virus and antibodies. In their work poliomyelitis viruses and antibodies have been assayed in replicate suspensions of monkey kidney or Hela cells on the basis that recognizable pH differentials develop between infected and uninfected suspensions due to cell respiration differences.

The present authors have reported that vesicular stomatitis virus (VSV) multiplies and produces cytopathogenic changes in guinea pig kidney and bovine tongue tissues

TABLE I. Yields of Vesicular Stomatitis Virus, New Jersey Type, in Tissue Cultures and Cell Suspensions.

	Description of cultures and suspensions*				Log TC ID_{50} per ml†					Virus yield ratio,‡ culture/suspension
					No. hr incubation at 37°C					
Exp. No.	Method§	Kidney	Vol, ml	Medium	0	24	48	72	96	
1	C	Guinea pig	2	NF	—	*6.45*	5.07	6.20		.32
	S		2	"	—	4.95	5.45	*6.95*		
2	C	"	2	"	3.70	*6.87*	4.87	3.53	2.00	2.20
	S	"	2	"	3.70	5.20	6.30	*6.53*	6.20	
3	C	"	2	"	2.53	*6.20*	5.70	3.10	2.00	8.53
	S	"	2	"	2.53	3.33	5.20	4.29	*5.33*	
	S	"	200	"	1.53	2.00	2.73	4.33	*5.20*	
4	C	"	2	"	2.53	*6.20*	4.30	2.87	2.33	2.89
	S	"	2	"	2.53	3.53	5.08	*5.53*	5.53	
	S	"	2	"	3.53	3.45	*5.87*	5.53	5.20	
	S	"	200	"	2.53	2.53	4.53	*5.87*	5.20	
	S	"	200	"	3.53	3.33	5.53	*5.70*	5.33	
5 (a)	C	"	2	"	3.47	4.98	*5.30*	4.47		.42
	S	"	200	"	3.47	3.62	4.78	5.15	*5.68*	
(b)	C	"	2	TF	3.47	4.62	*5.70*	4.62		7.95
	S	"	200	"	3.47	4.47	4.68	*4.80*	4.52	
6	S	"	200	"	2.53	2.50	2.83	*4.25*	4.17	
7	S	Bovine	200	"	3.80	4.47	4.93	*5.98*		

* NF and TF, see *Materials and methods.*

† Initial values of TC ID_{50} calculated from titers of inocula and dilutions effected. Italicized values represent maxima.

‡ Maximum TC ID_{50} for culture divided by that of the suspension. In Exp. 3 and 4, TC ID_{50} values employed for suspensions are averages of the maxima.

§ C = Culture; S = Suspension.

grown *in vitro*. It was also observed that this virus inhibited respiration in suspensions of the trypsin-dispersed kidney cells(7). This presumptive evidence for the growth of VSV in cellular suspensions has been confirmed in the present report and data have been obtained on the comparative yields of virus from surviving guinea pig and bovine kidney cells in suspension and from guinea pig kidney following its *in vitro* cultivation.

Materials and methods. Cell preparation. Young guinea pigs weighing approximately 250 g served as a source of kidney tissue. Bovine kidney obtained at an abattoir was cooled to approximately 4°C and brought to the laboratory. The kidneys were cut into small fragments and then digested at 37°C with 0.25% trypsin (1:300 Nutritional Biochemicals Corp.) contained in the phosphate buffered saline (PBS) described by Dulbecco and Vogt(8). After 10 minutes the supernatant fluid of the digest was decanted through 3 layers of gauze. Fresh trypsin solution was added to the undigested residue and the digestion and decantation processes were repeated until most of the kidney tissue was consumed. The combined cellular filtrates were centrifuged at 1,000 rpm for 5 minutes and the sedimented cells were then washed three times in PBS at 600 rpm for 2 minutes. The cells were finally suspended in nutrient fluid, 1 part packed cells to 200 parts of fluid. The nutrient fluid used most extensively consisted of Hanks' balanced salt mixture, horse serum and chick embryo extract, 5:3:2; in Table I and the text, this fluid is referred to as medium NF. A fluid consisting of Hanks' salt mixture, horse serum and hydrolyzed bovine plasma albumin (5% aq. Travamine, Baxter Laboratories), 95:3:2, herein referred to as medium TF was used in some instances. Penicillin G, streptomycin sulfate and phenol red were added to give concentrations of 100 units/ml, 0.1 mg/ml and 0.005%, respectively. *Cell suspensions.* Aliquots of the trypsin-dispersed cells in nutrient fluid were introduced into culture tubes (16 mm dia. x 150 mm) or 1 liter Erlenmeyer flasks, 2 ml and 200 ml, respectively. After inoculation with virus the cell suspensions were incubated at 37°C. Tubes were held in roller drums rotating at 12 rph and each flask was shaken at one vibration per second in an upright position on a shaking machine having a one-inch stroke. Aliquots of the infected suspensions were assayed in tissue cultures for viral content every 24 hours over a 4-day period. The samples assayed represented pooled 0.05 ml aliquots from each of 10 tubes while those from flasks were single unpooled aliquots. *Tissue cultures for virus production.* Two-ml volumes of the trypsin-dispersed cells in nutrient fluid were introduced into culture tubes. These were slanted and incubated at 37°C. After 4 or 5 days the fluids were changed and at 7 days the cultures contained confluent outgrowths of epithelial cells and fibrocytes. Each of 10 cultures was inoculated with virus and at 24-hour intervals a 0.05 ml sample of infected fluid was removed from each tube for pooling and subsequent virus assay in tissue cultures. *Tissue cultures for virus assay.* These were similar to those used for virus production except that in their preparation 0.5 ml rather than 2.0 ml of the cell suspension was introduced into each tube. The assay cultures were used from 8 to 11 days after preparation. Fluid changes were made at 4 or 5 days and again just before use in titrations. In preparation for the bioassays serial 10-fold dilutions of the infectious fluids to be tested were prepared in PBS. One-tenth ml volumes of the viral dilutions were inoculated into the assay cultures, 5 cultures per dilution. After 48 hours incubation, infection of the cultures was read on the basis of cytopathogenicity and the cumulative 50% infectivity doses for tissue culture (TC ID_{50}) were calculated by the Reed and Muench method(9). *Virus.* Virus-containing fluids for inoculation of cell suspensions and cultures were those obtained after 35 or more consecutive passages of VSV, New Jersey (N. J.) type, in guinea pig kidney cultures. Tissue culture ID_{50} values were determined for all except the first used inoculum.

Results. The data in Table I reveal that the yields of virus from guinea pig kidney cell suspensions (Expts. 1-5) ranged from 4.80 to 6.95 log TC ID_{50}/ml for both media and that the lower limit was 5.20 for NF media only. This compares with a nearly identical range of 5.30 to 6.87 log units for virus produced in guinea pig cultures. A second trial (Expt. 6) with guinea pig cells in suspension in medium TF again resulted in a low yield of virus. On the other hand, bovine kidney cell suspensions in medium TF yielded a relatively high virus titer in Expt. 7. While the virus yield ranges obtained by both methods of production were nearly identical, the yield ratio column of Table I shows that for Expts. 3 and 5b the maximum concentration of VSV in cultures exceeded by approximately 8-fold that found in suspensions. The remaining four comparative experiments showed smaller differences in virus yields by the two methods.

Virus yields in cultures most often reached maximum values within the first 24-hour incubation period and declined precipitously (1.35 to 2.0 log TC ID_{50}/ml) within the next 24 hours. An exception is in Expt. 3 where a sharp drop was not revealed until 48 hours after the maximum yield had been recorded. Expt. 5 differs from the others in that the peak in virus concentration was not reached for 48 hours; the decline thereafter was again rapid.

In cell suspensions, the virus yields are not maximal until 72 or more hours after infection. In contrast to the rapid inactivation of virus which occurs in cultures immediately following peak concentrations, the maximum titers recorded in cell suspensions are not materially changed during the subsequent 24-hour period. Also, a 100-fold increase in suspension volumes did not alter the concentration of the virus in the harvested fluids.

Discussion. It is concluded that guinea pig kidney cultures or cellular suspensions infected with VSV, N. J. type, produce approximately equal quantities of virus when medium NF is employed. The recorded 8.53-fold greater yield of virus in culture than in suspension (Exp. 3) is moderated by the fact that the yields of virus produced in the suspensions of this experiment are at the lower limit of the yield range for the suspension method when medium NF is employed. Furthermore, the highest yield of virus in the present studies was produced in a cell suspension (Exp. 1), exceeding that from the culture of Exp. 3 by a significant amount. For each of the remaining trials with medium NF the yield ratios do not reveal significant differences in virus production. Three-fold differences in TC ID_{50} values are within the chance range expected in the assay of serial 10-fold dilutions of VSV, 5 cultures per dilution.

In the presence of medium TF, guinea pig kidney cultures appear superior to suspensions for VSV production in the one case studied (Exp. 5b). With this medium more virus was produced in suspension when guinea pig kidney cells were replaced by those from the bovine.

It is considered that the delayed production of virus in suspensions may have been concomitant with the possible initiation of cellular growth. However, repeated counts of nuclei by the method of Sanford *et al.*(10) carried out at 24-hour intervals over 5-day periods on uninfected cell suspensions in flasks failed to reveal any appreciable increases in cell concentrations. Quantitative studies which would relate VSV yields to cell numbers for both trypsin-dispersed kidney cells and their progeny in tissue cultures have not been carried out.

Summary. Vesicular stomatitis virus, New Jersey type, was propagated in suspensions of surviving trypsin-dispersed kidney cells of guinea pigs as well as in cultivated epithelial cells and fibrocytes. Both production methods gave nearly equal virus yields, the highest being approximately $10^{6.9}$ tissue culture ID_{50}/ml. The highest virus concentration in cultures and suspensions were most frequently recorded at 24 and 72 hours following infection, respectively. Cell suspensions were increased in volume from 2 ml to 200 ml without altering the per ml virus yields. After peak virus concentrations had been reached, the rate of destruction of virus infectivity upon further incubation was significantly higher for cultures than for suspensions. Vesicular stomatitis virus was also shown to grow in suspensions of dispersed bovine kidney cells.

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Endocrine Factors in Pathogenesis of Experimental Poliomyelitis in Hamsters.* Role of Inoculatory and Environmental Stress. (22206)

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Golden hamsters are susceptible to intracerebral but refractory to intraperitoneal inoculation of MEF$_1$ virus(1,3). Susceptibility of intracerebrally inoculated animals undergo significant alterations as a result of natural or experimental disturbances in the adrenal-testes endocrine equilibrium(2). This paper reports the effect of interacting stress, endocrine function, and route of inoculation upon poliomyelitis susceptibility in hamsters.

Materials and methods. Male Golden hamsters 25-30 g were inoculated with MEF$_1$ virus or control sterile mouse brain emulsion (SMBE) in equal volume. Animals were killed in groups of 20, daily for 5 days, then at 7, 10, 12, 15, and 28 days following inoculation. Weights of adrenal glands, testes, thymus, and interscapular brown fat (ISB) of animals inoculated with virus were compared with those inoculated with the SMBE and with uninoculated controls sacrificed the same days. The following volumes of MEF$_1$ virus suspension, diluted 1/100 in saline, were used: 0.05 ml intracerebrally, 0.02 ml intraspinally, 0.5 ml intraperitoneally, and 0.2 ml intramuscularly and subcutaneously. Paralytic incidence and mortality were noted daily for 21 days. Animals dying without paralytic symptoms have been excluded. Histological examination of the spinal cord, and mouse inoculation were periodically used to control clinical observations. The experiments were repeated a number of times and the results were averaged per group, per season, and per year. Except for the investigation of climatic stress, all animals were kept in the constant environment of air conditioned rooms at approximately 23°C. The results were analyzed by statistical procedures previously described(2). The association between series, *i.e.*, proportion

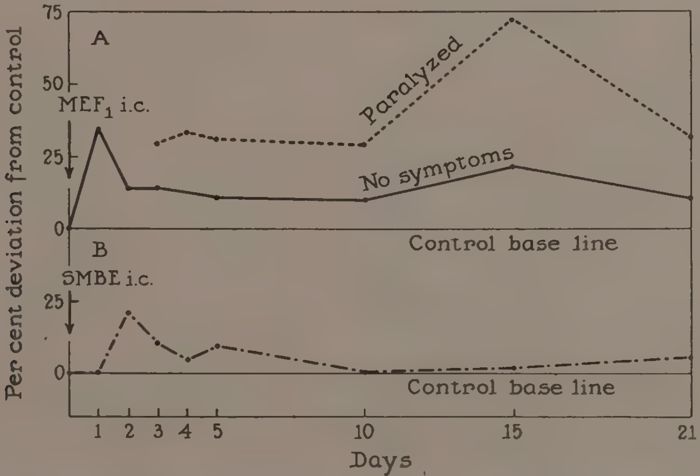

FIG. 1. Percent deviation from control of mean adrenal weight following intracerebral injection in Golden hamsters: A. in 206 animals injected with MEF$_1$ virus; B. in 625 animals injected with sterile emulsion (SMBE). Base line represents non-injected controls sacrificed the same time and in equal number as experimental animals.

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* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Effect of Preparatory Pathogen-Free Intracerebral Injection (SMBE) upon Susceptibility of Golden Hamsters to Poliomyelitis Virus (MEF_1) Inoculated Intraperitoneally.

Treatment		No. animals	Incidence of paralysis, %	Statistical significance "P"
Intracerebral	Intraperitoneal			
Sterile M.B.E.* .05 cc	MEF_1 virus 5 × .5 cc	125	16.00	.0001
None	*Idem*	254	3.93	

* M.B.E. = Mouse brain emulsion.

of poliomyelitis incidence (Y axis) and proportion of hypertrophic adrenals (X axis), under varying stressing conditions, was analyzed by using the correlation technic(18,19).

Results. Effects of inoculatory trauma. The effect of intracerebral inoculation upon the endocrine glands was studied on 1500 hamsters. Animals inoculated with virus intracerebrally averaged significant adrenal hypertrophy within a 5-day period following injection (Fig. 1). Alterations of the average adrenal weight noted beyond this period were statistically insignificant. Average weights of thymus, testes, and ISB showed no significant changes within the initial 5-day period. Endocrine alterations of the same pattern were found when SMBE was substituted for the viral inoculum (Fig. 1), indicating that the observed adrenal hypertrophy was produced by the intracerebral inoculation, rather than by MEF_1 virus. Among animals inoculated with virus, adrenal hypertrophy was found in the paralyzed animals regardless of elapsed time between intracerebral inoculation and start of paralysis (Fig. 1). To determine whether the refractory state to intraperitoneal inoculation virus could be eliminated by a preliminary sterile intracerebral injection, animals were injected intracerebrally with SMBE, after which MEF_1 virus was given intraperitoneally every other day, 3 or 5 times. Poliomyelitis, confirmed clinically and pathologically, developed in 12-25% of the treated animals. Control groups, repeatedly inoculated with virus intraperitoneally, only developed the disease in 2-6% (Table I).

Persistence of MEF_1 virus in various inoculated tissues. A single intraperitoneal inoculation of virus given the same day as the sterile intracerebral injection did not produce poliomyelitis. This suggests that rapid disappearance of the virus precluded dissemination before initiation of the endocrine alterations which usually appear 48 hours after the intracerebral trauma (Fig. 1). To determine whether there is any significant difference in persistence of poliomyelitis virus within the cranial and peritoneal cavities, several groups of infected animals were given a single injection of 5 mg cortisone at different intervals following virus inoculation. Cortisone produced

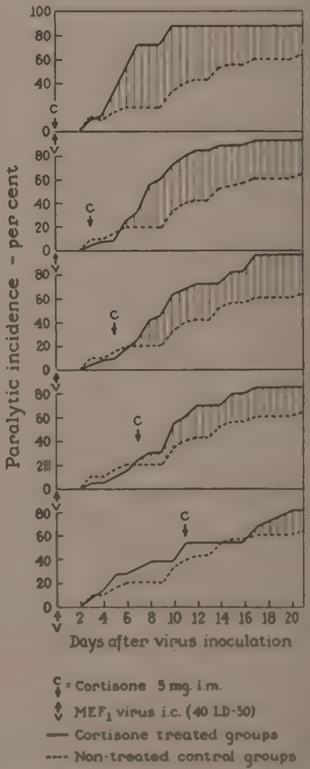

FIG. 2. Conversion of quiescent poliomyelitis into paralytic disease with the aid of cortisone given at varying intervals after intracerebral inoculation of virus.

TABLE II. Frequency of Hypertrophic Adrenals According to Age in Populations of Normal Hamsters Kept in Constant Environment of An Air Conditioned Laboratory.

Body wt, g	No. animals	Proportion of adrenals over 14 mg/100 g, %
25–35	403	17.61
35–50	538	19.70
50	135	46.66

enhancement of paralytic incidence as late as 11 days following intracerebral inoculation, indicating that potent virus was still present in a quiescent state. Conversely, no paralysis or death was produced by cortisone beyond the third day following intraperitoneal, intramuscular or subcutaneous inoculation of virus thereby suggesting that the virus was rapidly destroyed when introduced peripherally, unless endocrine alterations like those induced by a sterile intracerebral injection favor its progression and dissemination.

Endocrine reactivity to stress and susceptibility to poliomyelitis virus. Assuming that individual or seasonal variations in susceptibility to poliomyelitis may be associated with individual or seasonal deviations from the general pattern of endocrine reactivity to inoculatory trauma, adrenal reactivity was investigated and compared with susceptibility to poliomyelitis virus in 5000 hamsters maintained in the relatively constant environment of an air-conditioned laboratory. The weight of 14 mg per 100 g body was considered as the boundary limit between normal and hypertrophic adrenals. This represents the median figure between the mean value found in 299 hamsters submitted to normal climatic fluctuations and the mean adrenal weight of 486 animals kept in air-conditioned rooms and sacrificed during the same seasonal periods as the former. Actually, more than 80% of 941 young hamsters (25-50 g), kept in a constant environment and autopsied at various periods of 1954, showed adrenals weighing less than 14 mg/100 g. The frequency of adrenal hypertrophy increased with the age, due possibly to repeated occasional stresses associated with daily care, *i.e.*, feeding, cleaning, etc. (Table II).

When SMBE was injected intracerebrally in young hamsters, 33% of 327 animals sacrificed during the following 5 days, showed adrenal hypertrophy. When poliomyelitis virus was similarly injected in 372 animals, 35% developed paralytic symptoms during the first week of observation. Table III shows that seasonal variations observed in adrenal reactivity to inoculatory trauma were paralleled by similar fluctuations in the paralytic incidence of the first week following inoculation.

As may be seen from Table III, the paralytic incidence of the first week following intracerebral inoculation of virus, represents only 2/3 of the total incidence recorded during a standard observation period of 21 days under constant environmental conditions. Based upon the assumption that the additional 1/3 paralytic incidence may be incidental to additional stresses, such as daily examination of the animals, 100 hamsters were inoculated with MEF_1 virus intracerebrally then separated into 2 groups. One group was examined routinely every day, while the other group was kept undisturbed for 21 days. Table IV shows that both morbidity and mortality were higher in the group examined daily, than in the non-disturbed group.

TABLE III. Seasonal Fluctuations in Adrenal Reactivity to Inoculatory Trauma as Compared with Parallel Variations in Susceptibility to Poliomyelitis Virus.

	Intracer. inj. S.M.B.E.*		Intracer. inj. of MEF_1 virus		
Seasonal period	No. animals	Incidence of adrenal hypertrophy, %	No. animals	Incidence of paralysis 1st wk, %	Total paralytic incidence 3 wk, %
5/54	99	31	89	38	61
9/	129	24	147	26	40
2/55	99	46	136	42	66
Yearly avg	327	33	372	35	55

* S.M.B.E. = Sterile mouse brain emulsion.

TABLE IV. Effect of Daily Examination upon Paralytic Incidence and upon Mortality from Poliomyelitis in Golden Hamsters Inoculated with MEF_1 Virus Intracerebrally, 50 Animals in Each Group.

Groups	Paralytic incidence, %	Mortality, %
Examined daily	64	44
Not disturbed	52	30

Though the difference was found statistically insignificant, it persisted when the experiment was repeated on 175 additional animals.

The role of environmental stress in enhancing paralytic incidence in intracerebrally inoculated hamsters is demonstrated in Table V. The paralytic incidence is significantly higher in animals kept in the variable environment of normally heated rooms than in animals kept in air-conditioned laboratories. A significant increase in belated paralytic incidence, *i.e.*, appearing beyond the first week following inoculation, seems to be the main effect of the stressing environment. This explains the paradoxical observation that, despite the greater morbidity, the mean incubation period was longer in a varying than in a constant environment due to the number of belated paralytic cases provoked by environmental stress (Table V).

Groups of animals maintained in refrigerated rooms at 5°C or dark rooms at 23°C showed adrenal hypertrophy in a percentage similar to the percentage of paralytic poliomyelitis observed under similar environmental conditions (Table VI).

Statistical analysis of the association between series in 7 pairs of groups totaling 1602 animals submitted to varying experimental stresses as shown in Tables III and VI, revealed a linear correlation of high statistical significance between the percentage of adrenal hypertrophy in one series and the percentage of paralytic poliomyelitis in the other. As a corollary observation, 82% of the animals developing poliomyelitis showed adrenals over 14 mg/100 g, at the time when the paralytic symptoms first became evident.

Comparative effects of various inoculation procedures. In hamsters kept in an air-conditioned environment, a sterile intraspinal in-

TABLE V. Progression of Paralytic Incidence following Intracerebral Inoculation of MEF_1 Virus in Groups of Hamsters Kept in Variable Environment as Compared to Groups Kept in Air Conditioned Rooms at 23°C.

Environment	No. animals	Early paralysis 1st wk, %	Significance "P" of difference	Late paralysis 2nd & 3rd wk, %	Significance "P" of difference	Total paralysis 3 wk, %	Significance "P" of difference	Mean incubation period, days
Variable (normally heated room)	600	26.16		35.50		61.66		9.40
Constant (air conditioned)	1333	33.53	.001	19.05	Highly significant*	52.58	.001	7.53

* .00000000001.

TABLE VI. Incidence of Adrenal Hypertrophy as Compared to Incidence of Paralytic Poliomyelitis in Groups of Hamsters Submitted to Various Environmental and Dietary Conditions for 3 Weeks.

	Adrenal reactivity			Susceptibility to MEF$_1$ intracer.	
Treatment	No. animals	Avg adrenal wt, mg/100 g	Incidence of adrenal hypertrophy, %	Total No. animals	Paralytic incidence, %
Refrigeration	139	15.14	61.39	86	65.11
Dark room	183	15.07	52.33	93	58.06
Protein enriched diet	125	14.17	54.40	132	59.84
Idem + S.M.B.E., intracer.*	144	14.76	52.77		

* Intracer. inoculation of .05 cc sterile mouse brain emulsion.

jection was followed by a larger proportion of adrenal reactions than an intracerebral one, *i.e.*, 46% as compared to 19% during April 1955. The incidence of paralytic poliomyelitis also was significantly higher in intraspinally than in intracerebrally inoculated animals. The mean incubation period was shorter after intraspinal than after intracerebral inoculation of virus (Table VII). An intraspinal injection with SMBE prior to and on the same day as the intracerebral inoculation of MEF$_1$ virus, raised the incidence of paralysis to the same level as in animals receiving virus intraspinally. The mean incubation period remained however, within the range of intracerebrally inoculated animals. A sterile intracerebral injection preceding administration of virus intraspinally did not change significantly the paralytic incidence or the incubation period as compared to controls inoculated only intraspinally (Table VII).

Adaptation to stress and susceptibility to poliomyelitis virus. Intracerebral inoculation of MEF$_1$ virus after varying periods spent in cold environment, resulted in a decreased incidence of poliomyelitis in proportion to the time spent in the cold environment prior to inoculation, *i.e.*, 70% when refrigeration started on the day of inoculation, 66% in groups refrigerated for 5 days prior to inoculation, and 57% in groups refrigerated for 10 days preceding the inoculation. Chronic adrenal hypertrophy was found in 86% of hamsters kept under protracted climatic stress. When poliomyelitis virus was injected intracerebrally in similar groups, paralytic incidence was only 66%, suggesting that, with time, adaptation had compensated for the enhancing effect of the adrenal hyperfunction in about 20% of these continuously stressed animals (Table VIII). Conversely, spontaneous adrenal hypertrophy was found in less than 20% of the animals in the air-conditioned environment, while paralytic incidence that followed intracerebral inoculation of virus exceeded 50% (Table VIII). As shown above, in such constant environment, 2/3 of the total paralytic incidence depends upon adrenal reactions induced by inoculatory trauma which takes over the role of the eliminated environmental stress.

In a stress environment, the enhancement of viral progression by inoculatory trauma seems to be reduced, as suggested by the fact that the incidence of early paralysis was significantly lower in an inconstant, than in air-

TABLE VII. Effect of Inoculatory Trauma upon Paralytic Incidence and upon Mean Incubation Period in Hamsters Receiving MEF$_1$ Virus Either Intracerebrally or Intraspinally.

Inoculation						
Intracer.	Intraspinal	No. animals	Total paralytic incidence, %	Early paralysis 1st wk, %	Late paralysis 2nd & 3rd wk, %	Mean incubation period, days
MEF$_1$ virus	None	82	39.02	28.04	10.97	6.50
None	MEF$_1$ virus	77	68.83	68.83	0	3.33
MEF$_1$ virus	S.M.B.E.*	78	66.66	48.71	17.94	6.98
S.M.B.E.	MEF$_1$ virus	77	62.33	55.84	6.49	4.45

* S.M.B.E. = Sterile mouse brain emulsion.

TABLE VIII. Effect of Normal Climatic Fluctuations upon Adrenal Weight, Susceptibility to Poliomyelitis Virus and Mean Incubation Period in Hamsters as Compared to Controls Maintained in a Constant Environment.

		Spontaneous adrenal hypertrophy			Susceptibility to MEF_1, intracer.			
Period of inoculation	Environmental conditions	No. animals	Average adrenal wt, mg/100 g	Proportion adrenals over 14 mg /100 g, %	No. animals	Paralytic incidence, %	Mortality, %	Mean incubation period, days
4/ 1/53-5/15	Variable*	60	17.09	86.66	139	66.90	38.84	9.39
4/ 8/54-5/14	Constant†	58	12.16	20.68	73	61.64	36.98	5.76
9/ 1/53-10/30	Variable	179	17.17	78.20	218	65.13	36.69	8.80
9/13/54-10/19	Constant	156	11.72	20.50	158	49.36	20.25	5.97
11/13/53-11/18	Variable	32	15.70	53.12	86	61.62	27.90	10.70
11/ 5/54-11/19	Constant	95	11.87	9.46	128	59.37	23.43	7.98

* A normally heated room. † Air conditioned at 23°C.

conditioned environment, despite a total paralytic incidence significantly higher in the former than in the latter (Table V). The paradoxical reduction of early paralytic cases in the fluctuating environment might have resulted from a reduction in the number of adrenal responses to inoculatory trauma, due either to temporary exhaustion of reactivity or to adaptation caused by the repeated environmental stress preceding the inoculation.

Effect of diets. A diet enriched with 1% casein hydrolysate, in addition to the normal Rockland mouse diet, increased the incidence of adrenal hypertrophy from 10% in the controls to 50% suggesting either a direct effect of the diet, or an enhancement of the adrenal reactivity to minor environmental stresses (20). The same diet enhanced significantly the paralytic rate following intracerebral inoculation of virus. As in the case with environmental stress, the casein enriched diet increased the incidence of belated paralysis to 27% from 16.76% in controls and also slightly increased the mean incubation period.

Discussion. The above findings emphasize the importance of 4 factors in the pathogenesis of experimental poliomyelitis: (a) inoculatory or environmental stress, (b) endocrine reactivity to stress, (c) persistence of inoculated virus, and (d) correct experimental timing. The effect of stress depends upon endocrine reactivity. Increased reactivity due to seasonal or dietary conditions enhances, while adaptation or temporary exhaustion of endocrine reactivity diminishes the effect of stress upon susceptibility to poliomyelitis. On the other hand, due to compensatory phases in the evolution of an endocrine disturbance, susceptibility varies with the timing of inoculation, in relation to the endocrine status present at time of viral inoculation (2). Some effects of cortisone in poliomyelitis infected hamsters give suggestive information regarding the mechanism by which the adreno-cortical hyperfunction induced by stress, may influence viral progression. Thus extensive spread and multiplication of virus in certain tissues is associated with the suppression of the non-specific inflammatory barrier produced by cortisone (1,4-9,11). Cortisone also favors accelerated and protracted viral multiplication in the brown fat cells in association with alterations in lipid metabolism (3,10,11). Experiments in progress demonstrate that stress enhances significantly the multiplication and persistence of MEF_1 virus in the brown fat of hamsters and also has a belated cortisone-like effect upon lipid metabolism. This effect upon persistence of virus may partly account for the high proportion of belated paralytic cases observed in a stress environment.

Cortisone induces viremia in hamsters by enhancing extraneural multiplication of virus, by suppressing inflammatory barriers, and by interfering with early antibody formation (1,8,9). According to Bodian, the viremia level is a main determinant of spinal cord invasion(12,13), and thus may account for the high paralytic rate observed in cortisone-treated animals.

Stress is also able to provoke viral invasion of the spinal cord. Thus, it has been shown that the paralytic incidence is significantly increased by a preliminary sterile intraspinal injection in intracerebrally inoculated, and by a preliminary sterile intracerebral injection in peripherally inoculated hamsters. A pathogen-free intramuscular injection increases paralytic rate and favors localization of the paralysis to the injected extremity of monkeys(12,13), and humans(16,17). This provoking effect of stress seems to be mediated by the resultant circulatory alterations which increase the blood supply to the corresponding part of the spinal cord(14,15) so that circulating virus can more readily penetrate into that region and set up an initial infection therein(13).

From studies thus far carried out, it may be concluded that properties like pathogenicity, invasiveness, etc., that are usually attributed to virus may actually depend on temporary alterations induced in the physiology of the host by factors as common as nutrition and environmental adaptation.

Summary. 1. The incidence of paralytic poliomyelitis in experimentally infected hamsters has been found to depend upon a variety of factors such as inoculatory trauma, environmental stress, and diet. A linear correlation was found between incidence of adrenal hypertrophy and incidence of paralytic disease induced by the above factors in 2 series of hamsters inoculated simultaneously, one with pathogen-free material, the other with MEF_1 virus. 2. Seasonal fluctuations in adrenal reactivity to the inoculatory trauma were closely associated with parallel fluctuations in susceptibility to poliomyelitis. Either, adaptation to chronic environmental stress, or rapid disappearance of the virus from certain inoculated tissues, interfered with the above correlation. 3. The mechanism by which endocrine glands and hormones may influence the progression of virus is discussed.

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Host Resistance to Bacteria in Hemorrhagic Shock IV. Effect of Hypothermia on Clearance of Intravenously Injected Bacteria.* (22207)

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Dogs recover if transfused after exposure to severe hemorrhagic shock of 2 hours duration(1). But they die if given an intravenous dose of bacteria which a normal dog readily destroys(2). This is true whether the bacteria are given during the two hour period of shock or anytime within the next 24 hours. This is evidence that hemorrhagic shock impairs the bactericidal mechanisms. In this report we present evidence that precooling the animal to be subjected to hemorrhagic shock protects the bactericidal mechanisms.

Method. Healthy mongrel dogs, weighing 15-22 kg were premedicated with morphine (2 mg/kg), and then immersed in iced water under light ether anesthesia until the rectal temperature reached 28°C. After a short interval for desaturation of the ether, the dogs were removed from the iced water and put into hemorrhagic shock by the elevated reservoir technic(3). No anesthesia was required thereafter to maintain the hypothermia. During the hypotensive period the rectal temperature ranged between 28° and 23°C. In the occasional instance in which it fell below 23°C warm water bags were applied. After 2 hours in shock the shed blood was returned by slow venous infusion, during which the dog was immersed in warm water until the rectal temperature reached 37°C. 5-50 billion *E. coli* or hemolytic coagulase-positive staphylococci (24 hour culture) were given intravenously either during the period of hypotension (Group IV, Table I) or after transfusion and warming to normal body temperature (Group V, Table I). In addition to these experiments, concurrent experiments were performed to test the response of normothermic dogs, transfused after exposure to 2 hours of shock, to the same bacterial suspensions. The animals were observed until death or full recovery.

Results. These are given in Table I. The data of Groups I to III are taken from the published report of the previous work in normothermic dogs referred to above(2). The additional experiments like those in Group III, performed concurrent with those in Groups IV and V, resulted in death in every instance.

In contrast to a zero survival rate for normothermic shocked dogs challenged with bacteria, the precooled shocked dogs (Groups IV and V) challenged the same way show a survival rate of 75% or better.

Normal dogs (Group I) show negative blood cultures 24 hours after injection of the bacteria, and the tissues are sterile within 48 hours. All dogs in Group III show a bacteremia at this time and until death, which occurs within 1-4 days. Of the survivors in Groups IV and V half showed positive blood cultures after 24 hours, but all showed negative blood cultures after 48 hours. All dogs which died in Groups IV and V showed positive blood cultures until death.

Discussion. From the Group IV experiments, in which bacteria were injected during hypothermia, one could attribute the result to some degree to the suppression of bacterial activity by the hypothermia. But the Group V experiments demonstrate that whether this is so or not, the antibacterial defense is sufficiently preserved by the hypothermia to enable the dog to cope with a dose of bacteria, injected after the body temperature is back to normal, which the uncooled dog cannot withstand.

In a forthcoming publication(4) further data are offered showing that the protection conferred on the anti-bacterial defense mechanisms by this degree and duration of hypothermia, though considerable, is not suf-

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* Supported by a contract with the Office of the Surgeon General, U. S. Army, through Subcommittee on Shock of the National Research Council.

TABLE I. Effect of Hypothermia upon Resistance to Bacteria Given during or after Shock of 2 Hours Duration.*

Group	Experiment	No. of dogs	Survivors No.	Survivors %
I	*E. coli* intravenously in normal dogs	10	10	100
II	2 hr shock in normothermic dogs	10	9	90
III	2 hr shock in normothermic dogs, and *E. coli* intrav. during shock or up to 24 hr after transfusion	30	0	0
IV	Hypothermia (28°C) induced prior to 2 hr shock, *E. coli* intrav. during hypotensive period—warmed to normal temp. after transfusion	8	6	75
V	Same as Group IV except *E. coli* given after body temp. was back to normal†	14	11	79

* Hemorrhagic shock—bleeding to blood pressure 30 mm Hg for 2 hr followed by transfusion of all shed blood.

† This group includes 4 experiments in which a coagulase-positive hemolytic *Staphylococcus aureus* was used. Recovery rate is about the same for this organism as for *E. coli* in normothermic and hypothermic dogs.

ficient to fully sustain their original potency.

Conclusion. The anti-bacterial defense mechanisms of the dog, which are severely injured by exposure to hemorrhagic shock of two hours duration, are protected to a considerable degree by precooling to 28°C.

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Fluorine in Urinary Tract Calculi.*† (22208)

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The pathogenesis of urolithiasis is one of the unsolved problems in modern medicine. A recent survey answered by 340 American urologists showed an average recurrence rate of 13.7%(1). It is apparent that urinary tract calculi form an inportant disease entity and that any new facts which may help clarify the problem of calculogenesis are worthy of attention.

The purpose of this note is to report that fluorine has been found in urinary calculi with frequency. Searching the literature failed to reveal reports of urinary calculi analyzed for fluorine. It has been reported that renal damage, albuminuria and hematuria have been found in humans and animals suffering from fluorine poisoning(7-13).

Methods. Parts of the stones from 10 cases of urolithiasis were analyzed for calcium, ammonium, magnesium, oxalates, phosphates, urates and carbonates in our laboratories. Other parts were analyzed for fluorine by the laboratories of Dr. H. Amphlett Williams of London, England, and Dr. Chester A. Snell of Foster D. Snell, Inc., N. Y. City. Determination of fluorine in such materials is a difficult, delicate and tedious laboratory procedure accomplished by different methods by each individual. The method used by Dr. Williams is that described by him in 1946(2). Dr. Chester Snell states(3) that his methods in determining fluorine content of calculi were those mentioned in the "Official Methods of Analysis

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TABLE I. Clinical and Laboratory Data.

Age	Sex	History	Location and No. of calculi	Analysis	Ca content (%)	Fluorine (parts/million) Williams	Fluorine (parts/million) Snell
66	♂	Prostatic hypertrophy 1 yr	Recurrent vesicle (2)	Uric acid, calcium oxalate	Not done	340	
46	♀	1 yr	Ureteral (3); vesicle (2)	Calcium oxalate and phosphate	" "	790	816
80	♂	5 yr of bilateral nephrolithiasis, 1 yr vesicle calculi	Vesicle (35)	Calcium carbonate and magnesium ammonium phosphate	26.9	210	315
72	♂	2 yr prostatic hypertrophy	" (100+)	Uric acid, calcium and ammonium carbonate and phosphate (trace of magnesium)	13.6	570	850
57	♂	1 mo	Ureteral (1)	Calcium oxalate	24.9	240	520
4	♂	Congenital bilateral ureterovesicle anomalies	Bilateral ureteral and vesicle (numerous)	Magnesium ammonium phosphate	0	30	35
72	♂	Several years prostatic hypertrophy	Vesicle (numerous)	Uric acid and calcium oxalate	5.9	19	0*
62	♂	1 yr prostatic hypertrophy	Small prostatic (numerous)	Not done	26.9	730	1290
68	♂	2 yr increasing prostatic hypertrophy	Large vesicle (2)	Inner layer uric acid Outer " " "	0 0	5 4	0* 0
37	♂	17 yr recurrent renal calculi	Ureteral (1)	Not done (previous calculi reported as calcium oxalate)	26.3	1560	1790

* Less than 1 part/million.

TABLE II. Fluorine Data from the Literature (for Comparison).

Material	Fluoride content, parts/million	Source
A. Human		
1. Normal teeth and bone	10–30	4†
2. " tissues	.8	4
3. a) 24 hr urine collection	.3–.5*	4
b) Normal urine	.4	5
4. Kidney (normal)	.78	5
5. " (in fatal fluorine poisoning)	11.6	5
6. Liver (normal)	.6	5
7. " (in fatal fluorine poisoning)	12.2	5
B. Exp. tissues from poisonings of dogs		
1. Acute fluorine poisoning	14–16	4
2. Chronic " "	2–5	4
C. Water		
1. N.Y.C. water supply	.1	14
2. Artificially fluoridated water	1.0	
3. Sea water	3.0	3
4. Naturally fluoridated water (very high content)	10 (Aztec, Ariz.)	6

* mg. † Ref.

of the Association of Official Agricultural Chemists," 7th edition. It is because of the difficulty of obtaining close correlation in results of the fluorine determinations that portions of all calculi, if available, were sent to both Dr. Williams and to Dr. Snell. Numerical differences reflect the difficulty in obtaining an accurate quantitative result in this analysis. It is also possible that fluorine content may differ at various parts of a calculus. This point will need further elucidation.

Table I gives a summary of clinical and laboratory data of 10 cases of urinary tract calculi studied.

Table II presents data from the literature, which show that values found in calculi greatly exceed those hitherto reported in biological materials including those obtained in experimental poisoning. The highest fluorine content in tissues reported in any of the papers is in the neighborhood of 30 parts per million; while in the calculi analyzed, fluorine contents up to 1500 to 1700 parts per million were found.

From the data on specimens in which calcium content could be determined, it will be noted that when calcium content is high, fluorine values are also high. In the two calculi in which no calcium was found, fluorine content is low.

One factor that all these patients had in common was urography. Almost all patients with calculi have had either intravenous or retrograde urography usually performed with one type or another of radio-opaque medium. In order to eliminate the iodine-containing urographic dyes as the source of the fluorine found in the calculi, samples of 3 of these materials were analyzed and found to contain no fluorine.

Summary. Fluorine has been found in high concentration in 8 out of the 10 urinary tract calculi analyzed.

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Lactic Acid Response to Epinephrine in Experimental Liver Disease.* (22209)

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Normal and partially hepatectomized rats were studied to determine whether differences in the utilization of lactic acid following the injection of epinephrine could be correlated with liver mass. Significant differences were observed and additional studies were carried out on the effect of carbon tetrachloride poisoning and bile duct ligation on the ability of rat liver to handle similar lactate loads.

Method. Sprague-Dawley albino male rats weighing 180-210 g were used. All animals were kept in a constant temperature room on a uniform diet. Animals received a subcutaneous injection of 0.02 mg/100 g body wt., of a freshly diluted solution of epinephrine with added glutathione (Adrin, Sharp & Dohme). Tail vein samples were used for lactic acid determinations. Partial hepatectomy was performed by a technic in which 70% of the liver mass was removed. Carbon tetrachloride poisoning was accomplished by the intraperitoneal injection of 0.10 cc of CCl_4/100 g body wt., a modification of a recently described technic(1).

TABLE I. Effect of Epinephrine on Blood Lactic Acid in Normal and Partially Hepatectomized Rats.

Hr after epinephrine	Normal rats	Part.-hep. rats
Resting, before epinephrine	17 ± .25* (57)	16.5 ± 1.3 (17)†
1	41 ± 1.7 (36)	46 ± 3.3 (16)
2	21 ± .3 (28)	37 ± 2.3 (17)
3	17 ± 2.3 (25)	36 ± 1.4 (11)
4	—	27 ± 2.3 (11)
5	—	20 ± 1.5 (13)

* S. E. † No. rats.

Results. Resting levels of blood lactic acid were the same in the normal and partially hepatectomized animals (Table I). Both

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* Aided by the James Hudson Brown Memorial Fund of Yale University.

TABLE II. Blood Lactic Acid Levels Three Hours after Epinephrine in Rats.

	No. rats	Blood lactic acid (mg %)
Normal	25	17 ± 2.3*
Part.-hep.	11	36 ± 1.4
CCl_4 2nd day	34	34 ± 3.5
" 5th "	14	20 ± 1.5
Bile-duct ligation 2nd day	8	14.5 ± 1
Idem 5th "	9	30 ± 2.5

* S. E.

groups exhibited a similar prompt rise in the lactic acid levels one hour after the epinephrine injection. Two hours after epinephrine, normal animals showed a rapid clearance of lactic acid and by 3 hours had returned to resting levels. Partially hepatectomized rats 1-4 days postoperatively did not return to previous resting levels of lactic acid until 5 hours after epinephrine. This difference was not present 9 days after operation. These results suggest that lactic acid determinations 3 hours after epinephrine could serve as a means of detecting liver damage. This was tested in rats by comparing CCl_4 poisoning and bile duct ligation.

The clearance of lactic acid was impaired in rats which had been injected with CCl_4 40 hours previously (Table II). These results were similar to those seen with removal of 70% of the liver mass. Determinations done 120 hours after CCl_4 poisoning, however, revealed a normal ability to handle endogenous lactic acid loads. These findings are consistent with the demonstrated regenerative capacity of the liver after CCl_4 intoxication(1).

Two days of biliary obstruction did not affect the ability to clear lactic acid in spite of obvious jaundice (Table II). Determinations done 5 days after duct ligation, however, showed impairment of this aspect of liver function probably related to the secondary cellular damage which follows prolonged bile stasis.

Discussion. When the role of the liver in lactic acid metabolism was established, investigators studied the resting blood lactic acid level in diseased liver states. Their results are equivocal(2,3). It was suggested that exogenous lactate loading might reveal impaired hepatic function, and much work has been done with this technic. These studies have proved that hepatocellular disease is detectable by this means(4,5). Few studies on endogenous lactic acid loading following epinephrine in liver disease have been reported. An early paper by Loeb *et al.* failed to show significant differences in the maximum elevation of lactic acid in patients with and without liver disease(6). Our results in rats show that while there is no difference in the maximum lactic acid elevation, there is a delayed return to normal levels following experimentally produced liver disease. These results are strikingly similar to those recorded by Nitzesco and Gontzea(7).

Theoretically the observed alteration in lactate accumulation might represent failure to destroy epinephrine by the damaged or absent hepatic tissue, since it is known that epinephrine is rapidly inactivated by the liver. In these experiments, however, the subcutaneous route of administration would minimize the opportunity for this epinephrine to pass through the liver. Furthermore, lactate disappearance curves of the same basic shape were obtained when epinephrine was injected by the intraperitoneal route despite a presumably high rate of entry into the hepatic circulation.

Summary. 1. Blood lactic acid levels were determined in rats subjected to liver damage by partial hepatectomy, carbon tetrachloride poisoning and bile duct ligation. 2. Hepatocellular loss or damage impaired the clearance rate of endogenous lactic acid after epinephrine. Biliary obstruction, *per se,* had no such effect.

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Comparative Thromboplastin Activity of Acetone-Dehydrated Rabbit and Human Brain.* (22210)

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The clotting factor present in various tissue extracts is commonly designated as thromboplastin and its potency is usually measured by the one-stage prothrombin time. With the development of prothrombin consumption time(1), an alternative method for estimating thromboplastic activity became available. Plasma from a severe hemophiliac which is almost completely devoid of thromboplastinogen and, therefore, of thromboplastic activity can be employed as assay medium. Prothrombin consumption in this plasma resulting from addition of various tissue extracts can be regarded as a measure of their thromboplastic activity. To make the procedure independent of hemophilic plasma, which is not always obtainable, a substitute was sought. Serum from normal platelet-poor plasma was found fairly satisfactory. It appears to contain all essential clotting factors except fibrinogen and thromboplastinogen when prepared under carefully standardized conditions. The two tissues studied were acetone-dehydrated rabbit and human brain because they are used most widely as reagents for the one-stage prothrombin time.

Methods. Prothrombin time. The method as previously described was employed and thromboplastin was prepared by means of dehydration with acetone(2). *Prothrombin consumption time.* One ml of blood was transferred to test tube which was placed in water bath at 37°C. Fifteen minutes after formation of a solid clot, the tube was centrifuged 1 minute at 2000 rpm and replaced in water bath for 45 minutes. Prothrombin remaining in serum was determined by the one-stage test previously described(3). When testing activity of thromboplastin preparations, 0.05 ml of the extract was mixed with 1 ml of hemophilic plasma (or serum obtained from normal platelet-poor plasma) and prothrombin consumption determined as before. *Serum for assaying thromboplastic activity.* Human blood collected with silicone-coated syringes and needles was transferred to centrifuge tubes also treated with silicone. The tubes were placed in ice bath for 30 minutes and then centrifuged at 3°C in an angle centrifuge for 40 minutes at 10000 rpm. Plasma which was nearly platelet-free was carefully removed and transferred to glass container placed in a water bath at 37°C. As the fibrin formed, it was promptly removed by wrapping it about a glass stirring rod. After all fibrinogen was removed, the serum was placed again in water bath at 37°C for 10 minutes and then for 30 minutes at 3°C. During this period residual thrombin becomes neutralized and thromboplastinogen disappears almost completely. No detectable diminution of prothrombin, labile and stable factors and PTC (plasma thromboplastin component) was observed. It was stored at –20°C.

Results. Thromboplastic activity measured by prothrombin time of human and rabbit brain dehydrated with acetone. It was shown previously that physiological saline extracts of acetone-dehydrated rabbit brain varying in concentration from 1½ to 5% gave a prothrombin time of 12 seconds(4).

TABLE I. Effect of Varying Concentrations of Aqueous Extract of Acetone-Dehydrated Rabbit and Human Brain on the Prothrombin Time.

	Prothrombin time, sec.			
	Rabbit brain		Human brain	
Conc. of extract, %	Normal plasma	Dicumarol plasma	Normal plasma	Dicumarol plasma
.5	14½		14	
1	14	26	13½	32
2	12	26	12	30
4	12	28	12	30
6	12	28	13	34
8	13	36	14½	44

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† Visiting Fulbright Scholar on leave from Centre Regional de Transfusion Sanguine et de Recherches Hematologiques, Montpellier, France.

TABLE II. Variation of Thromboplastic Activity in Various Types of Thromboplastin Preparations When Tested on Normal and Stable Factor Deficient Plasmas.

Thromboplastin preparation		Prothrombin time, sec. Normal plasma	Plasma deficient in stable factor
Rabbit brain		12	45
Human "		12	50
Dog "		21	67
Pig "		23	68
Russell viper venom		14	13½
Commercial preparation	I	11½	25
Idem	II	19	27
"	III	14	32
"	IV*	12	50

* Made from rabbit brain.

The results recorded in Table I show that the same prothrombin time is obtained with 2 to 4% extracts of acetone-dehydrated human brain. At higher concentrations, human brain extract has a depressing effect which is somewhat greater than that of rabbit brain. This has also been observed by Stein and Abrahams(5). Both human and rabbit brain extracts give very nearly the same prothrombin times on plasma from patients receiving Dicumarol.

To compare thromboplastic activity of acetone-dehydrated rabbit and human brain with other preparations of thromboplastin, the prothrombin time was done with various types of thromboplastin using normal plasma and plasma from patient with stable factor deficiency. The results are recorded in Table II. It is to be noted that only acetone-dehydrated human and rabbit brain extracts gave comparable results on normal and stable factor deficient plasmas. Of various commercial preparations of thromboplastin, only one, which is made from acetone-dehydrated rabbit brain, gave results which were similar to those obtained on human and rabbit brain processed according to the technic of Quick.

Thromboplastic activity of acetone-dehydrated rabbit and human brain as tested by prothrombin consumption time. When increasing amounts of extracts of rabbit brain are added to plasma from a severe hemophiliac, a proportionate increase in serum prothrombin time occurs as shown in Table III. When serum, which is obtained from normal platelet-poor plasma, is substituted for hemophilic plasma, the results are very similar showing that the latter can be used successfully in place of hemophilic plasma.

On comparing the thromboplastic activity of rabbit and human brain by adding the same quantity of extract of each to the assay serum and determining resulting consumption of prothrombin, a similar but slightly greater potency was observed as shown in Table III. Brain extracts of other species have less thromboplastic activity than human and rabbit brain when measured either by prothrombin time or prothrombin consumption time and it can be seen from the results in Table IV that as prothrombin time increases, prothrombin consumption time decreases. Russell viper venom is the exception in that it produces a fairly short prothrombin time but does not cause high consumption of prothrombin. The poor thromboplastic activity of chicken brain can be attributed to species

TABLE III. Comparison of Hemophilic Plasma and Serum from Platelet-Poor Plasma as Assay Media for Thromboplastic Activity of Rabbit Brain Extract.

Conc. of rabbit brain, %	Prothrombin consumption time, sec. Hemophilic plasma	Serum from platelet-poor plasma	
0*	8	8	(8)
.2	9	9	(10)
.5	9	12	(18)
1	16	15½	(27)
2	25	20	(42)
4	34	31	(50)
6	38	35	(63)

* Figures in parentheses are the prothrombin consumption times when human brain extract was employed.

TABLE IV. Thromboplastic Activity of Various Tissue Extracts as Measured by Prothrombin Time and Prothrombin Consumption Time.

Source	Prothrombin time, sec.	Prothrombin consumption time, sec.
Rabbit brain*	12	36
Human "	12	39
Dog "	20	24
Pig "	23	19
Chicken "	57	9
Russell viper venom	14	17½

* A 2% aqueous extract was employed.

TABLE V. Effect of Heating on Thromboplastic Activity of Rabbit and Human Brain.

	Prothrombin time, sec.	Prothrombin consumption time, sec.: Normal platelet-poor plasma	Hemophilic plasma	Serum from platelet-poor plasma
Rabbit brain:				
Heated to 50°C	12	74	34	39
" " 60°C for 20 min.	27	28	8	15
" " " " 60 "	32	12	8	8
Human brain:				
Heated to 50°C	12	94	49	50
" " 60°C for 20 min.	16	62	18	19
" " " " 60 "	24	13	8	8

specificity since it is highly potent when added to avian blood(6).

Action of heat on thromboplastic activity of rabbit and human brain extract. No activity is lost when either human or rabbit brain is heated at 50°C but at 60°C a marked loss of activity occurs especially in rabbit brain extract(7). Human brain is more resistant to heat, and even after 20 minutes at 60°C, it still produces prothrombin consumption when added to hemophilic blood. (Table V).

Discussion. Acetone-dehydrated brain extract is admittedly a crude thromboplastin but when properly prepared either from rabbit or human brain, it has remarkably constant activity as measured by the one-stage prothrombin time. In the present study it has been shown that rabbit and human brain preparations also have almost the same activity when determined by their effect on prothrombin consumption test. Since no method is known for preparing a purified thromboplastin which has an activity equal or superior to rabbit brain, it seems desirable to accept the latter as a temporary standard of reference. Contamination with blood can be reduced to a minimum when rabbit brain is carefully cleared of visible blood vessels. The product is, therefore, devoid of labile and stable factors which shorten prothrombin time of plasmas in which these factors are deficient. The reagent is, therefore, particularly valuable when prothrombin time is employed for estimating the over-all prothrombin activity of plasma. This is especially important in controlling Dicumarol therapy since this drug diminishes both prothrombin and stable factor. High concentrations of either rabbit or human brain exert an inhibitory effect on prothrombin time but for the concentration recommended (200 mg in 5 cc saline solution), the error is negligible.

Rabbit brain extract heated to 60°C for 20 minutes looses its holothromboplastic properties but becomes a suitable reagent for differentiating hemophilia and hemophilia-like diseases from thrombocytopenia and thrombasthenia since its addition does not correct the hemophilic group but does correct the latter conditions. Human brain cannot be substituted since it is too heat-resistant.

Summary. Rabbit and human brains, when dehydrated with acetone, yield a product which has a high, constant and practically equal thromboplastic activity as measured by the one-stage prothrombin time and by prothrombin consumption time using hemophilic plasma as the assay medium. Serum prepared from normal platelet-poor plasma can be substituted for hemophilic plasma. Human brain extract differs from rabbit brain in that it has a greater inhibitory effect at high concentration and is more resistant to heat.

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Protective Effect of Ganglionic Blocking Agents on Traumatic Shock in the Rat.* (22211)

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The effectiveness of adrenergic blocking agents in reducing the mortality in rats subjected to traumatic shock has been described (1). Local anesthetics(2) and atropine (3,4), an anticholinergic agent, also have been reported to produce significant protection from mortality resulting from total body trauma in the rat. Since ganglionic blocking activity has been described as a property of both local anesthetics and anticholinergic agents(5,6,7), an appraisal of ganglionic blocking agents for the prevention of death due to trauma appeared justified. That ganglionic blocking agents of sufficient potency and duration of action significantly reduce the mortality associated with traumatic shock in the rat has been established in the present study.

Method. The method of inducing traumatic shock was that of tumbling in rotating drums, as described by Noble and Collip(8). The method was modified in that only one shelf was used per drum and the animals' feet were individually taped. White male Wistar strain rats weighing 300 to 500 g were used. The speed of the drums was set at 40 revolutions per minute and the total revolutions recorded on a mechanical counter activated by an electronic relay. Preliminary studies indicated that mortality was directly related to the total number of turns of the drums with a lethal trauma of 50% at 800 turns. Nine hundred turns was chosen as a standard trauma level in these experiments to assure a mortality of 50% or more. The average mortality of all untreated rats (192) reported here was 77.1%. Treated animals received drug injected intraperitoneally 15 or 30 minutes prior to drumming. The compounds were dissolved in 0.9% saline and the dosages administered as mg (base or cation)/kg. Following trauma the animals were observed continuously and times of death were recorded. Any animal dying during trauma or 15 minutes thereafter was considered an immediate death and a gross autopsy was performed. Any animal evidencing rupture of a major blood vessel or a viscus was discarded. All other animals dying in the ensuing 24-hour period were considered as delayed deaths. Animals alive at the end of 24 hours were considered as surviving indefinitely. One control rat was tested with each drug-treated rat. A total of 16 animals, constituting one experiment at one dose level, were subjected to the standard trauma on the same day. Although daily mortality in untreated groups of rats varied from 50 to 100%, the susceptibility to trauma was assumed to be comparable in each group of animals subjected to drumming in one experiment. The differences in mortality between treated and control animals in individual experiments were analyzed for significance by exact probabilities for a 2 x 2 contingency table obtained from 2 times the tabulated (½ P)'s from Mainland's table (9). The over-all analysis for significance of all dose levels of each compound causing a reduction in mortality and not showing evidence of drug toxicity was analyzed by the chi-square method corrected for continuity.

Results. Table I presents the results of treatment of rats prior to trauma with 4, 5, 6, 7-tetrachloro-2-(2-dimethylaminoethyl) -indolene bis-methochloride (chlorisondamine), 3-

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* Statistical evaluation of results was performed by Joseph L. Ciminera, Department of Biometrics, Sharp & Dohme.

Chlorisondamine was kindly supplied by Dr. F. F. Yonkman, Ciba Pharmaceutical Products Inc., Summit, N. J. Pentolinium was obtained through the courtesy of Dr. J. Seifter, Wyeth Institute of Applied Biochemistry, Radnor, Pa. TEA was supplied through the courtesy of Dr. A. C. Bratton, Parke, Davis & Co., Detroit, Mich. Mecamylamine was made available through the kindness of Dr. K. Pfister, Chemical Division, Merck & Co. Inc., Rahway, N. J. Hexamethonium was prepared in the Department of Organic Chemistry, Sharp & Dohme.

TABLE I. Effects of Pretreatment with Ganglionic Blocking Agents on Traumatic Shock in the Rat.

Compound	Dose, mg/kg	Controls D/T	Controls %	Treated D/T	Treated %	% survival above controls	Significance (P) Individual	Significance (P) Over-all
Chlorisondamine*	1.0	6/8	75.0	3/8	37.5	37.5	N.S.	$<.001$
	2.5	8/8	100.	2/8	25.0	75.0	.007	
	5.0	”	100.	3/8	37.5	62.5	.0256	
	10.0	5/8	62.5	5/8	62.5	0	—	
Mecamylamine*	1.0	7/8	87.5	7/8	87.5	0	—	”
	2.5	4/8	50.0	3/8	37.5	12.5	N.S.	
	5.0	7/8	87.5	0/8	0	87.5	.0014	
	5.0	6/8	75.0	1/8	12.5	62.5	.0406	
	10.0	5/8	62.5	3/8	37.5	25.0	N.S.	
Pentolinium*	.5	5/8	62.5	1/8	12.5	50.0	N.S.	”
	1.0	6/8	75.0	”	12.5	62.5	.0406	
	2.5	8/8	100.	4/8	50.0	50.0	N.S.	
	5.0	5/8	62.5	0/8	0	62.5	.0128	
	5.0	7/8	87.5	3/8	37.5	50.0	N.S.	
	10.0	6/8	75.0	4/8	50.0	25.0	”	
Hexamethonium†	5.0	4/8	50.0	1/8	12.5	37.5	N.S.	$<.01$
	10.0	6/8	75.0	3/8	37.5	37.5	”	
	20.0	”	75.0	2/8	25.0	50.0	”	
	30.0	5/8	62.5	7/8	87.5	−25.0	—	
Tetraethylammonium†	5.0	6/8	75.0	7/8	87.5	−12.5	—	
	10.0	”	75.0	6/8	75.0	0	—	
	20.0	”	75.0	”	75.0	0	—	
	35.0	8/8	100.	8/8	100.	0	—	
	50.0	”	100.	”	100.	0	—	

* Injected 30 min. prior to trauma. † Injected 15 min. prior to trauma.
D/T = No. deaths/No. tested.

methylaminoisocamphane hydrochloride (mecamylamine), pentamethylene-1:5-bis-(1-methylpyrrolidinium bitartrate) (pentolinium), 1,6-bis-(tri-methylammonium)-hexane diiodide (hexamethonium), and tetraethylammonium chloride (TEA). The specificity of these agents for producing ganglionic blockade has been established(10-14).

Significant protection from mortality induced by trauma was observed with one or more dose levels in at least two experiments with chlorisondamine, mecamylamine and pentolinium. Hexamethonium and TEA failed to provide significant protection in any one experiment. In an over-all analysis of significance for all dose levels providing protection with no evidence of toxicity, significant protection also was apparent with hexamethonium. TEA failed to provide any protection, possibly because of its known evanescent effect(15,16).

The influence of pretreatment with ganglionic blocking agents on total, immediate, and delayed deaths from drum shock is presented in Table II. These represent the pooled experimental results from the individual experiments in which statistically significant protection was produced by chlorisondamine, mecamylamine and pentolinium. While by far the greatest protection from death was apparent in those animals dying in the delayed death group, 50% reduction in mortal-

TABLE II. Reduction in Immediate and Delayed Deaths by Protective Doses of Ganglionic Blocking Agents.

Procedure	No. of rats	Mortality Total No.	Mortality Total %	Immediate deaths No.	Immediate deaths %	Delayed deaths No.	Delayed deaths %
Control	48	40	83.3	13	27.1	27	56.2
Treated	48	7	14.5	4	8.3	3	6.2
Reduction in mortality—%			68.8		18.8		50.0

TABLE III. Increased Mortality in the Immediate Death Group from Pretreatment with Toxic Doses of Ganglionic Blocking Agents.

		Mortality					
		Total		Immediate deaths		Delayed deaths	
Procedure	No. of rats	No.	%	No.	%	No.	%
Control	56	44	78.6	10	17.8	34	60.8
Treated	56	43	76.7	31	55.3	12	21.4
± % change in mortality			−1.9		+37.5		−39.4

ity, a surprisingly significant ($P = <0.05$) reduction in mortality of 18.8% in the immediate death group also was observed.

Drug toxicity usually was evident in individual experiments from an increased mortality above the control group in those animals dying immediately, or by a decreased percentage of the treated animals surviving when the dosage range was increased, or both. Table III presents the pooled results from all individual experiments where drug toxicity was evident. A significant increase in the percentage of treated animals dying in the immediate period during or following trauma was apparent, although the difference in total mortality between treated and control rats was not significant. The decreased mortality in the delayed death group was not significant in that it represented a shift in deaths, or numbers of animals, to the immediate group.

Discussion. That pretreatment with ganglionic blocking agents produced significant protection from the mortality of drum trauma provides additional support for the observation that blockade of the sympathetic nervous system during physical assault prevents the sequence of pathologic events ultimately resulting in death(1). The role of the parasympathetic division is more difficult to assess, since the well characterized cholinergic blocking agents produce some degree of autonomic ganglionic blockade(6,7,15,17). The lack of a protective effect of atropine at lower dose ranges that effectively block the peripheral parasympathetic system indicates that postganglionic discharge of this division plays a less important part in the processes initiated by trauma. That atropine reduces mortality only when given in relatively large doses of 20 to 50 mg kg(2,4) suggests that ganglionic blockade(6,7) was responsible for the observed protection. The protection observed from local anesthetics(2) may well, likewise, be the result of ganglionic blockade(5).

A critical dose range of the effective compounds was required to produce a significant degree of protection. Doses below the critical level provided insignificant or erratic protection. The higher dose levels tested caused a decreased protective effect due to addition of drug toxicity to the stress inflicted, even though such doses, in the absence of trauma, were below the lethal level. These observations have also been made with adrenergic blocking agents(1).

The finding that a significant reduction in mortality of those animals usually dying during trauma, or shortly thereafter, was achieved by pretreatment with ganglionic blocking agents indicates that common mechanisms may be involved in both early and delayed deaths due to trauma, or that the early deaths merely represent the more susceptible individuals in the test population. In contrast to this observation, an increase in the relative incidence of immediate deaths was noted with doses of adrenergic blocking agents that provided significant protection against drum shock(1).

The differences observed pertaining to the effects of drug treatment on early mortality between these and previous data(1) possibly are explainable on differences of duration of trauma. The duration of drumming in the studies presented was 23 minutes, as compared to 15 minutes of drum rotation (with two shelves per drum) as reported by Levy *et al.*(1).

Summary. 1. Significant protection from death due to drum shock in the rat has been demonstrated by intraperitoneal pretreatment with the ganglionic blocking agents, chlorisondamine, mecamylamine, pentolinium and hexamethonium. 2. TEA failed to provide protection from mortality due to trauma. 3. Significant protective effects were demonstrable only at certain critical dose ranges. 4. The results provide added support to the observations that blockade of the sympathetic nervous system affords protection from the lethal effects of physical trauma.

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Renal Interstitial Pressure in Normal and in Anuric Man: Based on Wedged Renal Vein Pressure.* (22212)

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Knowledge of the interstitial pressure of kidneys would be of special interest in patients with acute anuria, as suggested by Peters(1) and Oliver, MacDowell and Tracy (2), that anuria is due to raised interstitial pressure caused by interstitial edema. However, this suggestion has never been substantiated by measurements in man. In dogs suffering from a renal lesion induced by clamping the renal artery for 3 to 4½ hours, the pressure recorded in a needle inserted in the renal tissue was within the normal range(3).

The method of assessing interstitial, or capillary, pressure in human tissues by wedging a cardiac catheter into small venules of an organ has been applied to lungs and liver, but never to kidneys. Such a technic is presented in this report. From the finding of a normal wedged renal vein pressure (WRVP) in acute anuria following shock it is concluded that the anuria in such cases is not caused by raised interstitial pressure.

Methods and material. With patients in the supine position a cardiac catheter No. 7-9 (in a boy of 7 yrs. No. 4) was passed under fluoroscopic guidance into a renal vein, most often the right. After entering the renal vein the catheter was advanced as far as possible into the kidney veins. When the catheter was properly wedged, pressure measurements were performed with a condenser manometer (4) connected with a cathode ray oscillograph and a photographic recorder. At beginning of

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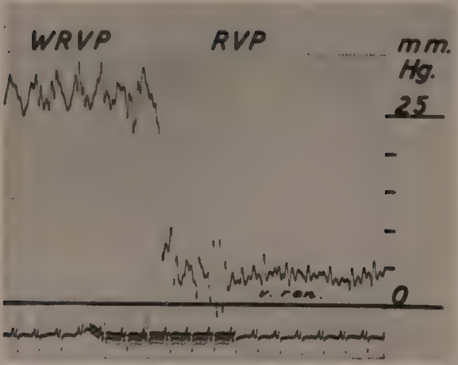

FIG. 1. Abrupt drop in pressure as catheter is withdrawn from a wedged position in renal vein to a free position.

withdrawal the catheter was stretched, followed by a sudden backward movement of the tip when released from the wedged position. Simultaneously with this characteristic slip a sudden and pronounced drop in pressure was seen together with an alteration of the configuration of the curve (Fig. 1). The highest pressure level was taken as the wedged renal vein pressure (WRVP), the lower level representing the renal venous pressure (RVP). In some cases it was impossible to obtain a pressure much higher than that in the main trunk of the renal vein. In other cases an unstable pressure was seen, presumably because the opening at the tip of the catheter was pressed against the wall of a vessel at a venous bifurcation, so that further insertion of the catheter in this position would alter the volume and pressure in the closed tube. It must therefore be emphasized that unless repeated reproduction of a pulsating curve which was stable at a high level and which showed the characteristic drop on withdrawal of the catheter, was obtainable at the oscilloscope, the examination was regarded as a failure. Besides WRVP and RVP, pressure in the right atrium and in the femoral artery were recorded. In all measurements the midaxillary line was used as zero reference. Pressure measurements were incorporated as part of renal function studies, which included renal blood flow in the anuric patients, extraction ratios of PAH and oxygen, and in some cases renal clearances(5). The 9 control subjects were patients with no evidence of renal disease or right sided heart failure. The 4 subjects with anuria or oliguria were patients who had developed acute renal failure after sustained shock following acute barbiturate poisoning (cases 3284, 3312, and 3395) or acute pancreatitis (case 3674). They were examined 5 to 7 days after the initiating shock when the peripheral circulation and blood pressure were normal, and the plasma urea concentration considerably elevated. In 2 cases follow-up studies were made.

Results. In the control group (Table I) WRVP ranged from 14-22 mm Hg, averaging 17.7. RVP ranged from 1.5-11.0, averaging 5.6 mm Hg. The right atrial pressures averaged 3.1 mm Hg, varying from −2-8. Arterial pressure averaged 125/79.

In patients examined during acute renal failure (Table II) the WRVP ranged from 15-30, averaging 20 mm Hg. RVP ranged from 5.5-15.0, averaging 10.5 mm. The right atrial pressures averaged 3.5 mm Hg, varying from 1-7. Arterial pressure averaged 128/76. In cases No. 3395 and 3674 follow-up studies showed no change in WRVP. The WRVP of 15 mm Hg given for case No. 3674 on 9th day of the disease, is from the right kidney. During the same examination a WRVP of 15.5 mm Hg was found in the left kidney.

Discussion. In the human kidney there is one central or internal system of veins—the arcuate veins—which drain the cortex as well as the medulla. Around the bases of the pyramids the arcuate veins anastomose; they unite in large branches—the interlobar veins

TABLE I. Wedged Renal Vein Pressure (WRVP) and Pressure in Renal Vein (RVP), Right Atrium and Femoral Artery in 9 Control Subjects.

Case No.	Sex	Age, yr	WRVP	RVP	Right atrium	Femoral artery
			(mm Hg)			
3454	♂	46	20.0	3.5	0	120/80
3509	♀	33	16.0	2.5	−2.0	"
3018	♀	19	18.5	1.5	1.0	115/70
1123	♀	30	18.5	5.0	1.0	100/65
3620	♀	32	18.0	6.5	5.5	120/70
3651	♀	40	16.0	3.0	1.0	145/90
3596	♂	27	16.5	11.0	6.0	158/84
3697	♀	46	14.0	8.0	7.0	140/90
3385	♂	7	22.0	9.0	8.0	110/80
Means			17.7	5.6	3.1	125/79

TABLE II. Pressure Measurements in 4 Patients with Acute Renal Failure Examined at Various Stages of the Disease. Symbols as in Table I.

Case No.	Sex	Age, yr	Days after shock	Days after onset of anuria	Urine flow, cc/24 hr	Plasma urea, mg %	24 hr endogenous creatinine clearance, cc/min.	WRVP	RVP	Right atrium	Femoral artery
								mm Hg			
3284	♀	41	7	5	170	444	.8	15.0	5.5	1.0	120/80
3312	♂	47	5	3	29	280	.5	30.0	15.0	5.0	162/82
			7	5	15	420	.1	18.0	13.0	—	93/70
3395	♀	27	5	4	85	380	1.0	21.0	9.0	7.0	162/85
			12	11	1740	638	4.8	22.0	10.0	10.0	182/96
			21	20	3020	46	66.0	27.0	3.5	2.0	125/82
3674	♂	51	6	5	700	405	6.0	16.5	10.0	1.0	102/63
			10	9	1680	260	33.0	15.0	10.0	—	122/80

—that run between the sides of the pyramids and the columns of Bertini to the neck of the calices, around which they form a second system of anastomoses much shorter and thicker than those at the base of the pyramids. The interlobar veins join through short thick stems into the main renal venous trunk(6,7). From necropsy studies of human kidneys we have the experience that in most cases a No. 8 cardiac catheter can be easily advanced through an interlobar vein towards its junction with the arcuate veins, the lumen of the interlobar vein being occluded by the catheter.

The pressure at the tip of a catheter occluding an interlobar vein peripherally to the set of anastomoses near the pelvis cannot be lower than the arcuate venous pressure. The pressure in an occluded interlobar vein cannot exceed the pressure in the arcuate veins, as the numerous anastomoses between these vessels are thought readily to drain away the blood which dams up behind the single catheter-blocked interlobar vein.

From these considerations it is reasonable to conclude that the WRVP as measured in this study is equal to the arcuate venous pressure, and hence very near to the pressure in the peritubular capillaries and interlobular veins. Therefore it seems justified that renal interstitial pressure can be assessed from WRVP. It is also seen that arcuate venous pressure is obtained whenever the tip of the catheter blocks the lumen of an interlobar vein between the 2 sets of anastomoses.

The normal value of 18 mm Hg for WRVP was in good agreement with values for arcuate venous pressure as measured by Swann *et al.* by renal vein catheterization in dogs; in these experiments a sudden drop in pressure of the same magnitude as observed in the present investigation (Fig. 1) was found when the catheter was withdrawn from an arcuate vein into an interlobar vein. Similarly, the present value for WRVP is nearly identical with the pressure measured in dogs by inserting a needle into the kidney(8,9,10,11).

It was impossible to demonstrate a raised WRVP in the 4 patients examined in the anuric or oliguric phase of acute renal failure. The follow-up studies in cases 3395 and 3674 deserve special interest, as they clearly demonstrate that the amount of urine excreted is independent of the WRVP.

Summary and conclusion. 1. A method is described for determination of wedged renal vein pressure (WRVP) in man. 2. In 9 normal subjects WRVP averaged 18 mm Hg. It was not possible to demonstrate a raised WRVP in 4 patients with acute anuria following shock, the average WRVP being 20 mm Hg. 3. It is argued that in agreement with experiences from lungs and liver, the interstitial pressure in kidneys equals the WRVP. The results suggest that in patients with acute anuria following shock the anuria cannot be explained by an increased interstitial renal pressure.

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Action of Diethylpropanediol and Diethylbutanediol on Isolated Sciatic Nerve and Ileum.* (22213)

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2,2-diethyl 1,3-propanediol (DEP) is a central nervous system depressant remarkable for its effectiveness in protecting animals against the convulsant action of pentylenetetrazol (Metrazol). Pharmacologically, it has been characterized as an interneuronal depressant with action throughout the neuraxis but with maximal effect in the brain stem (2,3,4). 2,2-diethyl 1,4-butanediol (DEB) which differs from DEP by but one methylene group is a convulsant remarkably similar to metrazol in action(5). It too has its principal action on interneurones throughout the central nervous system with a maximal effectiveness in the brain stem. DEP protects animals from the convulsant action of DEB. The available evidence indicates that this is a non-specific example of pharmacological antagonism since other depressants and stimulants act similarly in combination with the drugs in question.

It seemed of some interest to make a quantitative comparison of the effect of these two compounds on isolated axones and synapses.

Methods. Strength-duration curves were determined for desheathed rat sciatic nerve which was maintained in Tyrode's solution at 36-37°C in a nerve chamber. The nerve was allowed to equilibrate until the curve was reproducible. Increasing concentrations of drug in Tyrode's solution were added and the strength-duration curve was determined at 15 minute intervals until concentrations causing unequivocal depression of the nerve had been added. The nerve was then washed in drug-free Tyrode's solution until a normal level of excitability had been reached. In some experiments the excitability of the nerve was enhanced by calcium ion depletion by using a calcium-free Tyrode's solution and varying concentrations of sodium citrate as suggested by Brink *et al.*(6). As a simple method of evaluating ganglionic transmission, the contraction of the isolated rabbit ileum was used following the method of Feldberg(7). To study simple neuronal pathways in the central nervous system, the segmental reflex was studied in cats in which the spinal cord had been divided at the second cervical vertebra or at both the second cervical and last thor-

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TABLE I. Comparison of Rheobase of Isolated Rat Sciatic Nerve after Soaking in Drug.

Pre-drug, mM	DEB, 100%	DEP, 100%
4	117 ± 1	120 ± 4
8	122 ± 5	124 ± 4
16	135 ± 3	140 ± 2
32	180 ± 6	182 ± 6

Mean of 8 nerves ± S.E. of mean.

acic vertebra. The last lumbar or first sacral dorsal root was divided and placed on bipolar platinum electrodes. Stimuli of 0.05-0.2 msec. duration and less than 5 volt amplitude were delivered at 10 second intervals. The response was recorded from the corresponding ventral root from platinum electrodes by a RC coupled amplifier. Each drug was tested in 6 cats, 3 with high and 3 with both high and low spinal transection. DEB was given in 1 mg/kg doses repeated at 3-5 minute intervals and effects were usually seen when about 5 mg/kg had been given. DEP was given in a dose of 100 mg/kg; occasionally a second dose of 50 mg/kg was administered.

Results. In experiments on isolated desheathed rat sciatic nerve both DEP and DEB caused a reversible increase in rheobase with little change in chronaxie. The data presented in Table I show that both drugs were effective in causing an elevation in rheobase at 4 mM. Over a wide range, 0.001 mM-2.6 mM, no concentration of either drug was found which caused an increase in excitability.

Table II summarizes the experiments in which the excitability of the nerve was enhanced by depletion of calcium ion. At doses as low as 1 mM which caused no appreciable change in the threshold of normal nerve both the anticonvulsant and the convulsant drug caused a decrease in the hyperexcitable state.

TABLE II. Comparison of Percentage Increase in Rheobase on Calcium Deficient Isolated Peripheral Nerve.

	DEB, 1 mM (%)	DEP, 1 mM (%)
Pre-calcium	100	100
Post-calcium	37 ± 12	36 ± 10
Post-drug	88 ± 9	77 ± 10

Mean of 8 nerves ± S.E. of mean.

Spontaneous firing when present stopped after drug treatment when either compound was used in a concentration of 1 mM. The more dilute solutions of DEB or DEP were without effect.

In the experiments on rabbit intestine designed to test the action of the drugs on parasympathetic ganglia, both DEP and DEB had the same depressant action. Both compounds caused a decrease in the spontaneous motility of intestinal strips. As shown in Table III these effects were comparable at various dose levels.

TABLE III. Decrease in Spontaneous Contraction of Rabbit Intestine.

Dose, mM	% reduction DEP	 DEB
1	22 ± 1	18 ± 2
2	30 ± 2	22 ± 3
4	33 ± 2	26 ± 1
6	40 ± 1	40 ± 2

Mean of 6 strips ± S.E. of mean.

When 2 mM of DEB or DEP was added to the bath, 0.01 mM of nicotine or 0.4 mM/l of barium chloride failed to cause the usual increase in motility and tone. On the other hand, 0.025 mM of acetylcholine, 0.002 mM of pilocarpine or 0.005 mM of histamine still had significant stimulant effects. These findings are consistent with the view that the depression noted with DEP and DEB was indeed related to a ganglionic action.

In contrast to these results on isolated portions of the peripheral nervous system, the studies on the spinal cord confirmed the fact that DEP is a central nervous system depressant and DEB is a central nervous system stimulant. In 3 cats in which the spinal cord was divided at the level of the second cervical vertebra, DEP caused a depression of multisynaptic activity and some increase in the height of the monosynaptic spike. When the spinal cord was divided at the level of the last thoracic vertebra, DEP in each of the three cats caused a decrease in the height of both monosynaptic and polysynaptic response. Similar findings have been reported for mephenesin(8) and have been interpreted as evidence of a depression of interneural activity. The increase in the monosynaptic spike in the high spinal animals probably represents a release from inhibition.

DEB, 5 mg/kg, had an action which resembled that of strychnine(9). The decrease in monosynaptic response in the high spinal preparation associated with an increase in multisynaptic action suggests an increase in multisynaptic inhibitory action. In the three animals with the cord divided at a low level, the stimulant action is clearly demonstrated. Thus in a preparation which represents a sample of a segment of the central nervous system little influenced by diverse inhibitory and facilitory impulses, the stimulant action of DEB and the depressant action of DEP is clearly demonstrated.

Discussion. In the experiments on isolated axones and synapses taken from the peripheral nervous system, the action of DEP and DEB was depressant. The compounds appeared to be similar qualitatively and quantitatively. It is conceivable that different tests of axonal or synaptic functions might have demonstrated some difference but we do not feel that this possibility is very great. Studies of refractory period, accommodation, period of latent addition and so on did not seem to represent a profitable line of endeavor. In support of this point of view, it can be pointed out that cocaine which is a central nervous system stimulant has been shown to have the actions on peripheral structures that are associated with the "stabilizing" group of central nervous system depressants(1). At this point it seems reasonable to suggest that for study the cellular phenomena associated with the action of drugs on the brain and spinal cord methods utilizing the central nervous system should be sought.

Summary. 1. The action of 2,2-diethyl 1,3-propanediol (DEP), a central nervous system depressant with anticonvulsant activity and 2,2-diethyl 1,4-butanediol (DEB), a central nervous system stimulant with convulsant action have been compared on isolated synapses and axons. 2. Both compounds caused an elevation of rheobase of desheathed rat sciatic nerve at a concentration of 4 mM/l and no concentration tested caused an increase in excitability. Similarly, the treatment with each of these drugs of nerves rendered hyperexcitable with calcium ion deprivation showed only a depressant action 3. Experiments on isolated rabbit intestine showed that both compounds acted only as depressants. At 1 mM/l there was a depression of ganglionic transmission. 4. When the compounds were tested on simple reflex arcs in low spinal cats, DEP was depressant and DEB stimulatory.

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Presence of Hemolysin in Cultures of Pathogenic Leptospires. (22214)

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Hemolytic activity of a pathogenic leptospiral culture was observed in blood agar medium containing sheep erythrocytes when this culture was examined for bacterial contamination. The hemolytic potency of a "non-pathogenic" culture of water leptospires for various mammalian red blood cells had previously been reported by Sugimota(1). Except for Jungherr's casual reference to this phenomenon in reporting an outbreak of canine leptospirosis(2), no observations similar to Sugimota's has been reported for the pathogenic leptospires. Since toxic and hemolytic manifestations are frequently observed in leptospiral infections, further investigation of this chance observation was initiated by the authors—a study which demonstrated that a soluble hemolysin can be found in cultures of specific "serotype" strains of leptospires. Some characteristics of this soluble hemolysin will be described below.

Materials and methods. Preparation of hemolysin. Leptospires were cultivated in a 10% solution of inactivated normal rabbit serum (56°C for 1 hour) in isotonic phosphate buffered salt solution (pH 7.4) consisting of 0.667 g Na_2HPO_4, 0.087 g KH_2PO_4, 8.213 g NaCl, 0.190 g $MgCl_2 \cdot 6H_2O$ and 0.132 g asparagine dissolved in one liter of distilled water. Inoculated cultures were incubated 10-13 days at 30°C. Cultures were then centrifuged at approximately 4000 g, and the clear supernatant fluid removed and filtered through a Seitz E-K pad. Part of the supernatant fluid was stored in the refrigerator (5°C) and the remainder was frozen and maintained at –60°C. The sedimented leptospires were washed twice in phosphate buffer, resuspended with isotonic phosphate solution to 20% of the volume of the original culture and stored at about –60°C. For certain procedures, sedimented washed leptospires were disrupted by alternate freezing and thawing in dry-ice-alcohol and 37°C water baths, respectively. Cells were also disrupted when 20 ml aliquots of a 10-fold concentration of washed leptospires were subjected to sonic vibration at frequency of 10 kc and amplitude of 1.25 amps in a water-cooled magnetostriction oscillator. *Preparation of red blood cell (RBC) suspensions.* Sheep blood, collected by sterile venipuncture, was preserved in modified Alsever's solution(3). On the day of test, preserved cells were filtered through gauze, centrifuged at 1000 g for 10 minutes, washed 3 times with isotonic phosphate base and resuspended in a sufficient volume of base to produce approximately a 20% suspension. The concentration of cell suspensions was adjusted so that the complete lysis of a 1:40 dilution of RBC suspension had an optical density of 0.570 at 550 mμ. Erythrocyte suspensions from other animals were similarly prepared for one experiment. *Hemolysin test.* Two procedures were employed. In Procedure I, one part of a 20% sheep RBC suspension was mixed thoroughly with 9 parts of test substance in test tube and incubated in water bath adjusted to the desired temperature. After suitable intervals of time, aliquots were removed, rapidly centrifuged and diluted 1:4 with isotonic buffer. The percent hemolysis was estimated spectrophotometrically at 550 mμ. In later studies this procedure was discarded and a more sensitive indicator system (Procedure II) was substituted. In this procedure, 1 ml of test fluid was mixed with an equal volume of a 1% RBC suspension in a 10 x 65 mm cuvette. After appropriate intervals of incubation, cuvettes were centrifuged and direct readings of the optical density were taken. A hemolytic unit was defined as the reciprocal of the dilution of test fluid in a 1 ml dose which results in lysis of 50% of the red cells of 1 ml of a 1% sheep

TABLE I. Comparative Hemolytic Potency of Supernatant and Cellular Fractions of *L. hemolyticus* Cultures.

Culture	Fraction*	Milieu*	% hemolysis of dilutions Undil.	1:2	1:3	1:4	1:5	1:6	1:8	1:10	Units of hemolysin§
A (16-day-old)	S	b + r	90	94	93	66	62	38	17	6	5.5
	W (FT)†	b			93	71	48	17	8	6	.5
	W (FT)†	b + r	0	0	0	0	0	0	0	0	0
B (21-day-old)	S	b + r	86	85	88	80	71	40	7	7	5.6
	W (FT)†	b		29	0	0	0	0	0	0	<.2
	W (SV)‡	b		75	49	13	0	0	0	0	.3

* S = Supernatant; W = Washed cells; b = Base medium; r = Rabbit serum.

† 10-fold concentration of cells disrupted by successive freezing and thawing.

‡ " " " " " " sonic vibration.

§ Calculated/ml of original culture volume.

RBC suspension. The final volume of this test mixture is 2 ml. To determine 100% hemolysis and "normal" hemolysis of culture medium and diluent, suitable controls were set up with each test. *Preparation of antiserum.* A 2 kg rabbit was inoculated intravenously with successive doses of 5.0 ml, 5.0 ml, and 10.0 ml of filtered supernatant fluid at 5 to 6-day intervals. Seven days following the last inoculation, the rabbit was exsanguinated, the serum was collected, and stored at -20°C.

Results. Hemolytic studies were conducted on a strain of leptospires isolated from a human patient in Malaya. Upon antigenic analysis, the isolate was found to be a hitherto unrecognized serotype member of the hebdomadis group, and the strain was subsequently designated as *Leptospira hemolyticus* in this laboratory(4). Initial studies established the presence of a soluble hemolysin in cultures of *L. hemolyticus*. Treatment of a 2% suspension of sheep RBC with 12-day-old culture as well as with this culture's supernatant fluid, resulted in visible lysis of erythrocytes after a remarkably long incubation period of one to 2 hours at 37°C. On the other hand, a 10-fold concentration of washed leptospires prepared from the same culture and resuspended in uninoculated media demonstrated slight hemolytic activity after 6 hours of incubation. More definitive studies to determine the relative concentration of hemolysin in the culture supernatant fluid and in disrupted leptospires were conducted with two separate cultures of *L. hemolyticus*. In this procedure the more sensitive hemolysin test Procedure II was employed. The results of this study, shown in Table I, indicate that nearly all activity of *L. hemolyticus* cultures can be attributed to the soluble hemolysin present in the culture milieu. In addition, the presence of a RBC lytic inhibitor in rabbit serum is made evident by the lack of hemolytic activity of a suspension of disrupted cells in culture medium (buffered base + 10% rabbit serum). Even in the presence of RBC lytic inhibitors in cell-free liquor, the hemolytic potency of this fraction was 10- to 30-fold greater than comparable amounts of cells contained in the same volume of culture. Therefore, most of the subsequent work was conducted on the supernatant fluid.

The effect of 0°, 30°, 37°, 56°C temperatures on the hemolytic activity of the supernatant fluid, prepared from a 13-day-old culture of *L. hemolyticus*, was determined. In this study a 2% sheep RBC suspension was employed. After test tubes containing supernatant fluid were equilibrated in water baths of the desired temperatures for 10 minutes, sheep RBC were introduced and readings were taken at chosen intervals. The results shown in Fig. 1 indicate that the rate of hemolysis is considerably affected by temperature. At 0°C no hemolytic activity was manifested. The preliminary period before the onset of hemolysis, designated as the "induction period" by Bernheimer(5), was markedly reduced when the temperature was increased to 37°C. Hemolysin was completely inactivated at 56°C within 10 minutes.

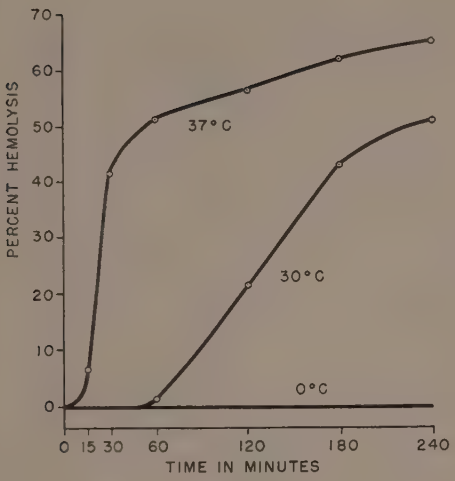

FIG. 1. Effect of temperature on course of hemolysis.

Additional studies of the culture supernatant fluid indicated that the hemolytic activity of the preparation was not reduced by continuous dialysis for 7 days against cold running tap water. No marked diminution of hemolytic activity was noted in the supernatant fraction, after this cell-free fluid was stored at 5°C (in the presence of air) or at –60°C during four months' observation. The hemolysin was not destroyed by lyophilization.

The hemolytic activity of the supernatant fraction of a culture of *L. hemolyticus* was tested against the RBC of the following species: sheep, horse, cow, goat, pig, dog, cat, chimpanzee, guinea pig, white rat, hamster, rabbit, human and chicken. Hemolysin test Procedure I was employed. Preparations were incubated at 37°C and readings were taken at the end of 2 and 4-hour intervals. Hemolytic activity was observed with sheep, cow and goat RBC only.

Forty-three different leptospiral strains representing multiple "serotypes" were screened to determine if the production of hemolysin was a generic or serotype-specific characteristic. Cell-free culture liquids were obtained from 13- to 14-day-old cultures of leptospires according to the methods previously outlined. When the spirochetes were harvested, the concentration of leptospires in each culture was determined spectrophotometrically on centrifuge-packed cells resuspended to effect a 10-fold concentrate of the culture's original volume. Supernatant preparations were treated with sheep RBC (Procedure I), incubated at 37°C, and the percent hemolysis was recorded at the 4th and 6th hour of incubation. The results of this study are shown in Table II. The minimum O. D. of any culture in which hemolysin was detected was 0.102. This figure was therefore chosen as the criterion for determining the amount of growth necessary to produce hemolysin. The results obtained with seven cultures showing lesser concentrations of leptospires were not considered to be significant. The production of hemolysin, demonstrated by the test procedures employed was restricted to specific serotype strains.

A close examination of the data in Table II revealed no apparent correlation between either the optical density or the length of the *in vitro* cultivation and the production of hemolysin by serotype strains. All strains employed were recognized "type" strains or strains identified in this laboratory through agglutinin-absorption procedures, with the following exceptions: Coke, Asbahadur, Campbell, Moulton, Perret, Williams, and Buxton. The disparity between the hemolytic reactions of various strains belonging to the same "serotype," such as, canicola, bangkinang, and hemolyticus, may reflect differences in the antigenic composition of these various strains, particularly in view of the fact that the serotype designations of some of these strains were based upon similarities in cross-agglutination lysis reaction patterns and not upon the more definitive agglutinin-absorption studies.

The results observed in various experiments indicated that the differences in hemolytic potential of *L. hemolyticus* cultures could be attributed to the age of the culture. Attempts were, therefore, made to determine the incubation period necessary to produce optimum hemolysin. A freshly seeded culture was distributed in 10 ml amounts in a

TABLE II. Hemolysis of Sheep RBC by Cell-Free Culture Liquids of Various Serotype Strains of Leptospires.

Serogroup	Serotype	Strain	Year isolated	Culture optical density	% hemolysis 4-hr	6-hr
icterohemorrhagiae	ictero. AB	Wijnberg	1926	.153	—	—
	new serotype A	DeGray	54	.148	—	—
javanica	javanica	Veldrat, Bat. 46	38	.115	—	—
schüffneri	schüffneri	ML-2	51	.110	—	—
	"	Bishop	54	.105	—	—
	"	Cake*	"	.132	—	—
	"	Asbahadur*	"	.115	—	—
canicola	canicola	Ruebush	48	.168	—	—
	"	Campbell*	54	.148	16	46
	"	Moulton*	52	.125	74	85
	new serotype	Jones	54	.152	—	—
benjamin	benjamin	Benjamin	37	.250	—	47
ballum	ballum	Pasteur	–	.129	—	—
pyrogenes	pyrogenes	Salinem	24	.183	—	—
	new serotype	Biggs	54	.136	—	—
cynopteri	cynopteri	3522	38	.102	7	78
sentot	sentot	Sentot	37	.225	—	—
autumnalis	autumnalis AB	Akiyami A	25	.165	—	—
	bangkinang	Perret*	54	.108	—	—
	"	Mason	"	.148	—	55
	new serotype	Williams	"	.180	—	—
djasiman	djasiman	Djasiman	37	.249	—	—
australis A	australis A	Ballico	"	.175	73	74
pomona	pomona	LC 73*	53	.214	48	74
	"	LC 78*	54	.145	62	76
	"	V-42*	52	.118	47	74
hebdomadis	medanensis	HC	29	.192	—	—
	Wolffi A	ML-34	51	.149	—	—
	hemolyticus	Marsh	54	.270	65	78
	"	Buxton*	"	.164	—	—
bataviae	bataviae	Van Tienen	32	.127	—	85
	djatzi	HS-26	51	.169	75	85
	paidjan	Quigley	54	.135	—	45
semaranga	semaranga	RS 173	37	.248	—	—
andaman	andaman	CH 11	31	.309	—	—
biflexa		CDC	–	.315	—	—

* Serotype designation was based on cross agglutination-lysis reactions.

series of 17 x 150 mm screw cap, chemically cleaned tubes. These tubes were incubated at 30°C. At specified intervals of time, the contents of 5 culture tubes were pooled, the supernatant fraction was then harvested, and the optical density of a 10-fold concentration of cells was determined as previously described. The supernatant fluid of harvested cultures was stored at –60°C. Tests, employing hemolysin Procedure II at 37°C, were conducted on the samples. When necessary, serial dilutions of supernatant samples were tested. The hemolytic potency of each sample was expressed in units of hemolysin. The results shown in Table III indicate that the hemolysin content of actively growing cultures increases with the increased concentration of leptospires and reaches maximum values at 1-3 days following optimal growth. Continued incubation of cultures reduces the hemolytic potency to approximately one-half the maximal value by the 50th day. This decrease in hemolytic activity may in large measure be due to thermal inactivation.

Studies on the effect of concentration of hemolysin on the kinetics of sheep RBC lysis were conducted with various dilutions of a supernatant preparation that was concentrated 2-fold by pervaporation and dialysis. Hemolysin Procedure I was employed and

TABLE III. Hemolytic Activity as a Function of Culture Growth.

Age of culture (days)	Culture optical density	Units of hemolysin/cc
0	.029	<1
1	.053	<1
2	.072	<1
3	.121	<1
4	.183	1– 4
5	.203	1– 4
6	.200	8–10
7	.209	10
8	.210	10
9	.214	12–16
10	.197	16–20
12	.192	16–20
14	.157	10
16	.161	10
18	.150	10
20	.133	8–10
24	.117	8–10
48	.109	8
50	.090	8

tests were conducted at 30°C. The plotting of percent hemolysis of various dilutions of the supernatant fraction as a function of time (Fig. 2) resulted in a series of curves which did not conform to the pattern generally observed with known hemolytic systems(5).

Attempts were made to determine the presence of specific hemolysin inhibitors in rabbit antiserum prepared against supernatant fractions (agglutination-lysis titer, 1:1600). The hemolysin employed was a 20-fold concentration of washed cells, disrupted by alternate freezing and thawing. The results summarized in Table IV indicated that normal rabbit serum inhibited lysis of sheep blood cells to the same degree as antiserum.

Discussion. The clinical symptomatology of the leptospiroses has been attributed to the toxigenic manifestations of these spirochetes (6,7). Gsell(7) maintains that leptospires produce a toxin with hemolytic properties and that the severity of disease reflects the intensity of intoxication. The hemolytic manifestations of leptospiral infections in man and animals have been frequently reported (6-9). The toxic effects in guinea pigs inoculated with preparations obtained from *L. icterohemorrhagiae* cultures have been demonstrated by Fukishima and Hosoya(10), and Higuchi(11), respectively. These observations, coupled with the fact that most, if not all of the known bacterial toxin products, other than the classic exotoxins, are hemolytic(12) strongly suggest that the hemolytic activity of leptospires is an attribute of a leptospiral toxin.

An appraisal of the experimental data in the light of current concepts of leptospirosis affords an opportunity to consider several factors that may operate in differential pathogenesis of various serotype strains for particular hosts. The various serotype strains show marked over-all differences in severity of disease produced in a particular host. Thus, in man, for example, mild, frequently subclinical, infections are generally produced by *L. pomona*, *L. mitis*, *L. grippotyphosa* and *L. hebdomadis* while the more severe icteric and hemorrhagic manifestations are more frequently observed in *L. icterohemorrhagiae*, *L. australis A* and *L. bataviae* infections(7). In addition, particular strains show discriminate pathogenicity for various mammalian hosts as exemplified by *L. pomona* infection in rats (inapparent), hamsters (lethal), guinea pigs (inapparent), humans (rarely icteric), swine (nonicteric), and cattle (frequently icteric) (6,7,9,13). Close examination of the data in which the hemolytic potency of 36 serotype strains, including 14 very recent isolates, was tested against sheep RBC (Table II) discloses differences among serotypes with respect to this characteristic. In some instances, *e.g.*, *L. icterohemorrhagiae*, Wijnberg, the absence of hemolysin in strains with

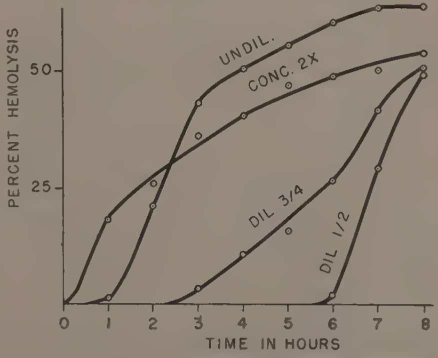

FIG. 2. Course of hemolysis of various concentrations of the supernatant fraction of a *L. hemolyticus* culture at 30°C.

TABLE IV. Comparative Titration of RBC Lytic Inhibitors in Specific Rabbit Antiserum and Normal Rabbit Serum.

Serum dilution	———Undiluted———			1:5	1:50	1:500	1:5000	—	—
" , ml	1	.6	.2	1	1	1	1	—	—
Buffer, ml	—	.4	.8	—	—	—	—	—	2
Hemolysin, ml	1	1	1	1	1	1	1	2	—
	Incubated test mixtures 30 min., 30°C								
1% sheep RBC, ml	2	2	2	2	2	2	2	2	2
	% hemolysis (1 hr 37°C)								
Antiserum	0	0	0	0	38	83	87	100	0
Normal serum	0	0	0	0	33	71	73		

a past history of "hemolytic" manifestations may perhaps be due to loss of this characteristic through continued *in vitro* subculturing over extended periods of time. In this respect, however, it is interesting to note that hemolysin was observed in 4 strains that were maintained in cultures for 17 to 23 years. The possible loss or maintenance of hemolytic properties after continued *in vitro* passage may perhaps reflect the varying degrees of adaptability of different strains to culture mediums, just as strains vary in their ability to maintain their virulence after repeated transfer *in vitro* medium. In our laboratory, a strain of *L. pomona* (S-91) was no longer lethal for hamsters after one year's passage in Fletcher's medium, while *L. djatzi* isolated from a human in Puerto Rico retained its lethality for hamsters after repeated transfers in this medium for 3 years. Van Riel observed an increase in pathogenicity for guinea pigs of an isolate after 8 months' cultivation in medium(14).

In addition to differences in the frequency of hemolysin-producing strains among heterologous serotypes, strain variation in the production of hemolysis is apparent within serotypes (Table II). Analogous observations of the differences in the pathogenicity of "homologous" serotype strains isolated from different sections of the world have been observed. The severity of *L. grippotyphosa* (bovis) infections in Israel and *L. bataviae* infection in Indonesia, are in marked contrast to the benign symptomatology produced by the respective European strains(7). On the basis of extensive studies of canine leptospirosis, Meyer *et al.*(15) concluded that—"The observations in California amply confirm the findings of Walch-Sorgdrager and Schüffner(16) that the icterogenic tendencies of *L. canicola* are a fixed property of certain strains." Stavitsky's(17) failure to substantiate the previously mentioned work of Fukishima, Hosoya and Higuchi, may be attributable to the toxigenic differences in the *L. icterohemorrhagiae* strains employed. In view of these observations, further consideration of the differences in pathogenicity of "homologous" strains may possibly be correlated in part with the hemolytic potential of these spirochetes.

The few species' RBC that are susceptible to the hemolysin of *L. hemolyticus* is particularly noteworthy. In contrast to our findings, Higuchi showed that "water leptospires" hemolysed rat, rabbit or guinea pig RBC. The data presented in Higuchi's report does not preclude the possibility that pathogenic leptospires were isolated. Whether or not this preferential activity is a function of the particular strain, reflecting perhaps the selective pathogenicity of that strain for various mammalian hosts, remains to be determined.

In large measure the activity of the hemolysin was masked by the presence of a relatively high concentration of rabbit serum (10%), necessary for the cultivation of leptospires. The inhibitory activity of normal rabbit serum on the lysis of mammalian RBC has been recognized for other hemolytic systems(18). The interfering activity of rabbit serum is demonstrated by data presented in Tables I and IV and from kinetic studies on the course of hemolysis of different concentrations of supernatant fluid (Fig. 2). The plotting of percent hemolysin as a function of time, employing different dilutions of the known bacterial hemolytic systems, elicits a typical consecutive series of sigmoid curves with decreased slopes and increased "induction periods" as the concentration of hemolysin decreases(5). The atypical series of curves shown in Fig. 2 is attributed to the differences in the kinetics of the hemolysin and inhibitor.

Superficially, the hemolytic potency of mature cultures of *L. hemolyticus* (Table III) is low when compared to the activity of cultures of beta streptococci(5). However, the hemolytic potential of leptospires is indeed considerable when one compares the extremely low yield of approximately 25-75 mg (dry weight) of leptospires obtained per liter of culture(19) with the several grams of streptococci obtained in an equivalent volume of medium(12). In addition, the hemolytic activity of leptospires is manifested in the presence of high lytic inhibitor (10% rabbit serum) concentrations.

Although it is interesting to consider our experimental data in terms of the current knowledge of the leptospiroses, definitive interpretation of the role of leptospiral hemolysin in the virulence of leptospiral strains and host reactions to infections must be approached with caution at this time. Further study of the nature and activity of leptospiral hemolysin and its mode of formation may provide clues to a better understanding of the factors involved in the pathogenesis of leptospiral infections.

Summary. A chance observation of the hemolysis of sheep red blood cells by a culture of leptospires was investigated. Studies were conducted on a newly disclosed serotype strain, isolated in Malaya and designated as *Leptospira hemolyticus.* The presence of a soluble non-dialysable, thermolabile, oxygen-stable hemolysin in the supernatant fluid of cultures was demonstrated. The optimum temperature for hemolytic activity was 37°C. At lower temperatures the activity of hemolysin progressively decreased. The hemolytic activity against sheep RBC occurred after a prolonged induction period. This phenomenon as well as the atypical kinetic activity of varying concentrations of hemolysin preparations were attributed to the inhibitory activity of the rabbit serum present in culture supernatants. The production of hemolysin was not a generic characteristic but was restricted to specific serotype strains. Hemolytic activity of *L. hemolyticus* was manifested against sheep, cow, goat RBC, but not against RBC of eleven other species screened. The antigenicity of this hemolysin could not be demonstrated. In actively growing cultures, maximum amounts of hemolysin were produced 1 to 3 days following optimum growth. The presence of a soluble hemolysin in specific serotype strains may figure significantly in explaining the pathogenicity of leptospiral infections.

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Inactivation of Some Animal Viruses with Gamma Radiation from Cobalt-60.* (22215)

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The need for obtaining better methods of inactivating viruses for preparing non-infectious vaccines and other biologicals indicates the desirability of exploring more efficient means of virus inactivation. While some information has been accumulated concerning the effect of small doses of alpha, beta and gamma radiation on viruses(1), little data that deal with sterilizing doses are available. Huber(2) has reported that many virus preparations can be inactivated by 40000 to 1400000 roentgen equivalent physicals (rep), using high intensity electron beams from a capacitron. Nickerson(3) has indicated considerably higher sterilization doses for viruses, varying from 1 to 20 million rep.

Studies described here deal with the effect of gamma radiation on 4 animal viruses *in vitro*. The experiments were designed to determine the dosage necessary to render these viruses non-infectious when irradiated in whole brain and in suspension. Since it has been suggested(1) that a relationship may exist between size of viruses and their resistance to ionizing radiations, the viruses used were selected to cover a range of particle size.

Materials and methods. At the time this study was initiated, poliovirus was selected as representative of a small virus. St. Louis encephalitis (SLE) and Western equine encephalomyelitis (WEE) viruses were chosen to represent medium sized viruses, and vaccinia a large virus. More recent data, however, indicate that poliovirus is somewhat larger than was previously believed and approximates the size of St. Louis virus(4). *Viruses* used and their approximate diameters were as follows: California strain of WEE virus 53 mμ(5), Hubbard strain of SLE virus 20-30 mμ(6) obtained from American Type Culture Collection. Armstrong 1166 neurotropic strain of vaccinia virus 225 mμ(7), Lansing strain of poliomyelitis virus 22 to 27 mμ diameter(4) were kindly supplied by the University of Michigan School of Public Health. All *virus suspensions* were prepared from infected brains of 3- to 4-week-old Swiss mice. Ten percent brain suspensions were prepared by grinding with alundum and cold physiological saline. Suspensions were subjected to the following cycles of treatment: (a) sedimentation at 2000 rpm for 5 minutes; the supernatant fluid was used as crude virus (CV), (b) sedimentation of CV at 3000 rpm for 10 minutes, (c) sedimentation of second supernatant fluid at 4000 rpm for 15 minutes, (d) filtration of third supernatant fluid through a Seitz EK pad, (e) sedimentation of the filtrate at 45000 rpm for 1.5 hours and reconstitution of the pellet in pH 7.2, 0.05 M phosphate buffer, (f) sedimentation of the reconstituted material at 4000 rpm for 15 minutes. Alternate high and low speed centrifugations were repeated until the virus preparation had been washed 3 times. Immediately after the final wash the virus pellet was resuspended in 2 ml of phosphate buffer to make a concentrated suspension of partially purified virus (PPV). One ml aliquots of all virus suspensions were placed in glass plastic-capped vials and shell frozen in a dry-ice-alcohol bath. Vials containing the frozen virus were then stored at –50°C until they were irradiated. Samples of whole mouse brains were frozen and stored in the same manner. *Titration of viruses.* The various virus suspensions were titrated for infectivity by intracerebral injection of 3- to 4-week-old mice using .05 M phosphate buffer as diluent. The LD_{50} titers were determined by the method of Reed and Muench(8). The various virus

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TABLE I. Rates of Inactivation of Poliovirus, St. Louis Encephalitis, Western Equine Encephalomyelitis and Vaccinia Viruses in Whole Brain, Crude Suspensions and Partially Purified States.

Virus	Diameter, mμ	Virus sample	Unirradiated controls	rep* radiation in millions							
				1	1.5	2	2.5	3	3.5	4	4.5
Poliovirus	22–27	WB†	4.3‡	3.3	3.0	2.3	1.9	1.3	0	0	0
		CV	6.0	4.6	4.0	3.3	2.6	2.2	1.4	0	0
		PPV	6.0	4.2	3.3	2.5	1.7	0	0	0	0
SLE	20–30	WB	7.2	5.8	5.1	4.3	3.5	3.1	2.2	1.5	0
		CV	6.0	4.6	4.0	3.3	2.7	2.1	1.4	0	0
		PPV	6.0	4.2	3.3	2.5	1.6	0	0	0	0
WEE	53	WB	8.1	6.3	5.4	4.8	4.0	3.2	2.3	1.6	0
		CV	6.0	4.4	3.6	3.1	2.2	1.6	0	0	0
		PPV	6.0	4.0	2.8	2.0	0	0	0	0	0
Vaccinia	225	WB	5.2	3.7	2.6	1.8	0	0	0	0	0
		CV	6.0	4.1	3.0	1.9	0	0	0	0	0
		PPV	6.0	2.5	0	0	0	0	0	0	0

* Roentgen equivalent physicals. † WB—whole brain, CV—crude virus, PPV—partially purified virus. ‡ All figures represent log of LD_{50} dilution.

suspensions were diluted to desired LD_{50}, just before they were irradiated. *Method of Irradiation.* Samples were removed from the deep freeze and placed in the center well of the large cobalt-60 source housed in the Fission Products Laboratory of the University of Michigan. Exposure rate of gamma rays emitted was approximately 200000 roentgen equivalent physicals (rep) per hour(9). One rep as defined for tissue in air represents an energy absorption dose of 93 ergs/g. Ferrous-ferric dosimetry was used for calibration. The calibration solution was placed in vials similar to those used for virus preparations and readings were based on oxidation of 15.4 μ moles of ferrous ions/liter/1000 rep. Uniform doses were attained by rotating samples on a horizontal plane in the center well of the Cobalt source. Samples to be tested were irradiated from 5 to 24 hours, during which time they were kept frozen with dry ice at approximately −72°C. All virus samples to be irradiated for a given experiment were placed in the center well at the same time. After the required exposure, samples were removed and immediately tested for presence of virus by intracerebral titration in mice, using 10 mice for each of an appropriate series of 10-fold dilutions. Control samples of virus were exposed to identical conditions but were not irradiated.

Results. As a first step toward studying the effect of gamma rays on animal viruses, samples of virus preparations were exposed to cobalt-60 radiations from 1 to 4.5 million rep. Frozen CV and PPV suspensions diluted (to the same volume) in 0.05 M phosphate buffer to contain the same number of LD_{50}, and whole brain preparations were irradiated under conditions described. During irradiation, samples of virus were removed at intervals computed to yield doses of gamma rays as indicated in Table I. Each sample was tested for reduction in virus titer by intracerebral injection in mice.

It may be seen that WB preparations for all viruses tested required longer periods of irradiation than did either CV or PPV suspensions. Crude suspensions of all viruses were inactivated at a significantly faster rate than were PPV suspensions. Results obtained with SLE virus are almost identical to those obtained with poliomyelitis virus. Comparison of these data show that both SLE and poliovirus required 3.5 to 4 million rep before CV suspensions were rendered non-infectious, and that PPV samples lost their infectivity at 2.5 to 3 million rep. From data presented it is evident that crude suspensions of WEE virus required less irradiation before infectivity was lost than did comparable suspensions of SLE or poliovirus. Similarly, PPV suspensions of Western virus were reduced in infectivity at a faster rate than were the SLE and Lansing viruses. Comparison of data on PPV suspensions reveals that West-

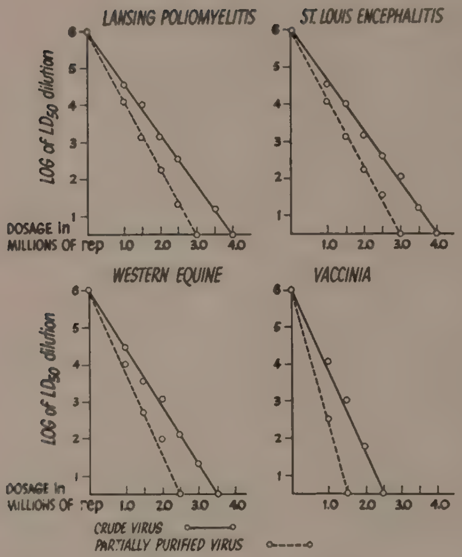

FIG. 1. Survival curves of gamma irradiated viruses in crude and partially purified suspensions.

ern PPV was no longer infectious after 2.5 million rep of irradiation. Partially purified SLE and poliovirus, on the other hand, required an additional 500000 rep before suspensions lost their pathogenicity for mice. It appears that inactivation of vaccinia virus by gamma radiation followed the same general sequence as that described for SLE, WEE, and poliovirus.

To show graphically the rates of inactivation for CV and PPV suspensions containing the same number of LD_{50}, the data in Table I were plotted on semi-logarithmic paper and presented in Fig. 1. The points on these curves are averages of virus dilution expressed as logarithms of the LD_{50}. The slopes of the curves suggest that purification of viruses removed some substance which protected the virus particle from the lethal effects of gamma rays. When the surviving virus fractions, or LD_{50}, are plotted on a logarithmic scale, the points are found to lie approximately on a straight line showing that, within error of the experiment, the surviving fraction is an exponential function of the dose. It should be pointed out that although error of assessment of virus activity may sometimes be rather large, there appear to be no constant deviations from exponential survival as distinct from random variations.

Discussion. The relationship between virus size and dose of gamma radiation required for inactivation is seen in Table I. The data show that CV suspensions of poliomyelitis and SLE virus required the same dosage of radiation before they were inactivated, while vaccinia virus was rendered non-infectious by a significantly smaller dose. Inspection of the data obtained with PPV suspensions suggest that smaller virus particles require larger doses of radiation for inactivation than do larger virus particles. It appears that the differential in size between poliovirus and SLE is not sufficient to make a difference in the dose of cobalt radiation needed for inactivation.

Of the PPV suspensions tested it may be seen that SLE and poliovirus with diameters of 20-30 mμ required 3 million rep for complete inactivation while the intermediate WEE virus required a slightly smaller dose. Vaccinia, the largest virus used, was inactivated with half the radiation required for the smallest viruses.

Preliminary experiments indicate that 2 to 3 times the dose of cobalt-60 required for inactivation does not impair the antigenicity of any of the viruses tested, and that gamma irradiated antigens elicit an immunologic response comparable to that induced by chemically inactivated viruses. These experiments will be presented in detail in a subsequent report.

Summary. 1. Gamma radiation from cobalt-60 proves to be an effective method for inactivation of Lansing poliomyelitis, St. Louis encephalitis, Western equine encephalomyelitis and vaccinia viruses. 2. Partially purified suspensions of viruses tested were more vulnerable to lethal effects of gamma radiation than were crude suspensions. 3. In crude and partially purified suspensions the smaller viruses required larger doses of gamma radiation for inactivation than did the larger viruses. 4. The rate of inactivation for viruses tested, within error of experiment,

is an exponential function of the dosage of gamma radiation.

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Infection of *Mansonia perturbans* and *Psorophora ferox* Mosquitoes with Venezuelan Equine Encephalomyelitis Virus. (22216)

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(Introduced by Morris Schaeffer.)
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Past studies have indicated Venezuelan equine encephalomyelitis (VEE) to be a mosquito-borne disease. In Trinidad, Gilyard(1) transmitted VEE from donkey to donkey by bite of *Mansonia titillans* (Walker). In Ecuador, Levi-Castillo(2) reported transmission by *Aedes taeniorhynchus* (Wied.), *M. titillans*, and *Culex quinquefasciatus* (Say). Recent work in the United States(3-5) has shown *A. triseriatus* (Say) to transmit efficiently in the laboratory.

The present report concerns studies on the VEE vector potentials of two additional species of mosquitoes, *M. perturbans* (Walker) and *Psorophora ferox* (Humboldt).

Materials and methods. The strain of VEE virus used, and methods of virus titration and of handling, feeding, incubating, and processing the mosquitoes were the same as reported previously(3,4). The *M. perturbans* were collected alive in Louisiana as adults, and were used in experiments 5 days later. The *P. ferox* were reared from larvae collected near Montgomery, Ala., and were 7-10 days old when used. Adult guinea pigs inoculated intraperitoneally with 1,000-10,000 mouse LD_{50} of virus 48 hours previously served as sources for mosquito infection. After incubation at 80°F, the mosquitoes were refed individually upon normal 3-week-old guinea pigs to determine their ability to transmit infection by bite.

Results. M. perturbans. Forty females engorged upon blood having a mouse intraperitoneal virus titer of $10^{8.4}$. A total of 16 egg rafts were laid by these specimens 6-13 days later. These rafts were ground in 2 ml of diluent each and titrated for virus. On the 13th day of incubation 16 of 29 mosquitoes remaining alive refed individually upon normal guinea pigs. These mosquitoes, as well as those which did not refeed, were then titrated to determine the amount of virus each contained. Table I summarizes the infection and transmission data.

P. ferox. Fifty females were fed upon blood containing virus titering $10^{6.3}$. Several hundred eggs laid 6-11 days later were pooled, ground in 2 ml of diluent, and inoculated into mice to test for virus.

On the 14th day of incubation the surviving mosquitoes were given opportunity to refeed individually upon normal guinea pigs. Subsequently, all mosquitoes were titrated to determine the amount of virus contained per

TABLE I. Infection of *M. perturbans* and *P. ferox* with VEE Virus.

Species	Titer of virus in blood ingested (mouse i.p. LD_{50})	% mosquitoes infected	Mouse i.p. LD_{50} contained/infected mosquito	% mosquitoes transmitting by bite	Virus recovered from mosquito eggs
M. perturbans	$10^{8.4}$	83 (24/29)	$10^{8.9}$ to $10^{6.0}$	38 (6/16)	+, 7 of 16 rafts*
P. ferox	$10^{6.8}$	84 (32/38)	$10^{1.5}$ to $10^{5.2}$	11 (2/18)	—

* No more than 300 mouse i.p. LD_{50} of virus were recovered from any of the rafts.

specimen. Infection and transmission results are included in Table I.

Discussion. The finding of virus in 7 of 16 *M. perturbans* egg rafts is of interest but as yet of unknown significance. Inability to rear this species in the larval stage prevented determining whether a second generation of infected females could be reared from these eggs. A similar study with eastern equine encephalomyelitis in *M. perturbans* has shown 2 out of 19 egg rafts laid following an infected meal to contain virus(6). It is possible, of course, that the virus may have been located only on the surface of the eggs despite rinsing with serum-saline solution. However, these findings are reported here in the hope that they may stimulate a more complete investigation.

The infection and transmission rates show *M. perturbans* and *P. ferox* to be susceptible to VEE infection and capable of transmitting it. Both of these species are abundant and widely distributed in the United States and are fierce biters upon man. Therefore, they must be considered as potentially dangerous vectors of VEE infection should this virus be introduced into the United States.

Summary. Two species of mosquitoes, *Mansonia perturbans* (Walker) and *Psorophora ferox* (Humboldt), were allowed to feed upon guinea pigs infected with the virus of Venezuelan equine encephalomyelitis. After 2 weeks incubation at 80°F, the infection rate and guinea pig transmission rate for *M. perturbans* were found to be 83% and 38%, respectively, and for *P. ferox*, 84% and 11%, respectively. Virus was isolated from 7 of 16 *M. perturbans* egg rafts, but not from the eggs of *P. ferox*. The significance of the infected eggs is still unknown.

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Host Resistance to Bacteria in Hemorrhagic Shock V. Mobilization of Phagocytes.* (22217)

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We have given evidence that hemorrhagic shock reduces efficiency of the phagocytosis-promoting factor in serum(1), increases sensitivity to bacterial toxins(2), and decreases the Properdin activity of serum(3). This report deals with the effect of hemorrhagic shock on the capacity to mobilize phagocytes to the site of challenge.

Method. 50 cc sterile beef infusion broth was injected intraperitoneally into rabbits immediately after transfusion for hemorrhagic shock of 2 hours duration. The infusion was also given at the same time to normal rabbits of the same weight and litter. Six hours later, after white blood count was made, both groups were killed by exsanguination. The peritoneal cavity was opened immediately thereafter, and cellular exudate recovered.† For this purpose the abdominal incisions were fashioned to produce a trough into which gelatin-Locke's solution at 4°C was introduced and aspirated after a short interval of gentle but thorough prodding of the immersed viscera. Washing and aspiration was continued until a total of 500 ml of Locke's solution was used. The recovered fluid was then spun in refrigerated centrifuge, and the precipitate washed 3 times in Locke's solution to get rid of fibrin and fibrin clots. The number of cells recovered was counted, and the percent of different types of cells determined from a count of 200 cells in stained smears. To compare number of cells mobilized in normal and shocked animals in response to the stimulus of the injected broth, it was necessary to obtain control data on number of cells that can be obtained by the same peritoneal washing technic from peritoneal cavities of normal and shocked rabbits of the same weight and litter, into which nothing has been injected. The shocked rabbits of this group were also killed 6 hours after transfusion.

Results. (Table I) A. *Control Data.* 1. *Normal rabbits—no intraperitoneal injection.* Number of cells recovered from 6 normal rabbits averaged 3.3 million (range 2.7-3.7 million). 65% of these were large mononuclear cells, 15% were lymphocytes and 20% were polymorphonuclear leucocytes.

2. *Shocked rabbits—no intraperitoneal injection.* Number of cells recovered from 6 shocked rabbits averaged 3.9 million (range 2.5-4.9 million). 75% of these were large mononuclear cells, 5% were lymphocytes, and 20% were polymorphonuclear leucocytes. The large mononuclear cells from both normal and shocked rabbits were elongated, somewhat flattened cells showing amoeboid activity. The fact that the number of cells in both groups was about equal, and that most of them were an apparently early form of the mature macrophage, suggests that they were for the most part traumatically desquamated peritoneal lining cells.

It is noteworthy that whereas no bacteria were observed in the cells from normal rabbits, ingested bacteria were seen occasionally in cells from 3 of the 6 shocked rabbits.

B. *Cellular Response 6 Hours After Intraperitoneal Injection of Beef Infusion Broth.* 1. *Normal rabbits.* The number of cells recovered from a series of 24 animals averaged 103 million (range 32-229 million). Of these 93% (range 85-98%) were polymorphonuclear leucocytes and 7% (range 15-2%) were macrophages, of which about half were mature, and the other half immature, *i.e.* like those recovered from the control animals. No bacteria were seen in stained smears of these cells. The white blood count in these rabbits

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† Sterile technic was employed in this and all further procedures where indicated.

TABLE I. Response of Normal Rabbits and of Rabbits Transfused after Two Hours Hemorrhagic Shock to Intraperitoneal Beef Infusion Broth Six Hours after Injection.

Intraper. stimulus	Cellular response in normal rabbits				Cellular response in shocked rabbits				Remarks
	% of				% of				
	Macrophages	Polys	Lymphocytes	Total in millions	Macrophages	Polys	Lymphocytes	Total in millions	
None	65*	20	15	3.3	75*	20	5	3.9	Bacteria in 3 of 6 shocked rabbits. None in unshocked rabbits.
Beef infusion broth	7†	93	—	103	75*	25		4.2	Twice as many bacteria as cells in 8 of 18 rabbits. None in unshocked rabbits.

* Chiefly immature macrophages. † 50% mature macrophages.

just before they were killed varied from 5800-6300 per cu mm.

2. *Shocked rabbits.* Twenty-four rabbits were prepared. Of these 6 died before the sixth hour following transfusion. Number of cells recovered from the 18 rabbits killed 6 hours after transfusion averaged 4.2 million (range 1.8-8.0 million if we except one from which 30 million were obtained). 75% of these (range 60-80%) were macrophages, most of which were immature, and 25% (range 40-20%) were polymorphonuclear cells. Just before these rabbits were killed their white blood cell counts were 3700-7600/cu mm.

In 8 of the 18 rabbits Gram positive and Gram negative bacilli and diplococci were found in considerable numbers.‡ There were about twice as many bacteria as cells per oil immersion field. Rather more than half of the bacteria were extracellular and the remainder intracellular. In addition *Coccidia* were observed in the exudate of 3 of the shocked rabbits.

Discussion. The considerable cellular response of the normal rabbit's peritoneal cavity to beef broth infusion demonstrates the irritant properties of this material. Since the number of cells that are recovered from the irritated peritoneal cavity of the shocked rabbit does not exceed the number recovered from the non-irritated cavity of the shocked rabbit, it is plain that shock severely impedes the mobilization of phagocytes, as Mahoney (4) and Miles and Niven(5) have demonstrated. That the cellular response should be poor *during* oligemic shock is not surprising, for the hemodynamic obstacle to mobilization is clear enough. But one would not expect this response in quantitative terms to be virtually zero during a 6 hour interval after restoration to a presumably normal hemodynamic status, in view of the fact that the white blood counts just before killing are about equal in normal and shocked rabbit. Since the normal animal's circulation delivers an average of 102 million polymorphonuclear leucocytes to the area of challenge, whereas the shocked animal's circulation delivers very few if any, the failure of these leucocytes in the shocked animal to enter area of challenge during post transfusion period appears to be due to some factor related to vascular permeability to these cells, rather than to a hemodynamic deficiency. This suggests that a mechanism such as the one recently described by Miles and Wilhelm(6), *i.e.* a cell permeability-promoting factor in serum, may have been inactivated by the shock process.

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‡ When clean but non-sterile technic was employed in harvesting the peritoneal exudate from normal rabbits, the only bacteria that we could recover were an occasional *B. sutilis*.

Another index of functional failure of the antibacterial defense system as a result of shock is inability of peritoneal lining cells and other precursors in the shocked animal to respond to the challenge for more macrophages, for the macrophages that are recovered are not increased in number, and of those recovered, most are still of the immature variety. This response contrasts with that of the normal rabbit in which the number of macrophages is about doubled, and about half of them are of the mature variety.

No bacteria were found in or outside the phagocytes in normal rabbit's exudate. But at least three varieties of bacteria, in a 2:1 proportion to cells, were found both in and outside the cells of the shocked rabbit.[§] Since the stimulus to migration of bacteria from the intestine cannot be considered to be any less in the normal than in the shocked rabbit, the presence of bacteria in the shocked animal is further and unequivocal evidence of collapse of the antibacterial mechanisms in shock.

Summary and conclusion. When the peritoneal cavity of the rabbit recovering from mild shock in response to transfusion is challenged by an irritant fluid, the cellular response as compared to that in a normal rabbit is extremely poor. The failure to mobilize polymorphonuclear leucocytes is, for the most part, not due to defective hemodynamics, but to a disturbance of vascular permeability to migrating leucocytes. The failure to supply additional macrophages at the site of the irritation appears to be due to a defect in the cellular processes involved in the conversion of their precursors. The presence of a substantial intracellular and extracellular bacterial population in the peritoneal fluid of the shocked animal, and the absence of bacteria in the peritoneal fluid of the unshocked animal, is further evidence of the loss of resistance to bacteria in the shocked animal, and of its persistence after restoration of a normal hemodynamic status.

[§] Previous studies indicate that the source of these bacteria is the intestine and that route of invasion is by transmural migration(7).

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Effect of a High Phosphorus Diet on Acid-Base Balance in Guinea Pigs.* (22218)

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The injurious effect of high phosphorus diets for the guinea pig has been reported (1,2). The animals grew slowly, developed mineral deposits in soft tissues and usually succumbed within a few weeks. More recently the ameliorative effect of magnesium and potassium on this syndrome has been described(3). Thus the guinea pig seems to be more sensitive to high levels of dietary phosphorus than the white rat and appears to have a considerably higher requirement for potassium and magnesium.

This report is concerned with one reason for the injurious effect of high phosphorus intake and the beneficial effects of potassium and magnesium.

Methods. The diets used were similar to those described by House and Hogan(3). The percentage composition of the basal ration (3884) was: acid-washed casein(4) 30 g; sucrose 47; celluflour 15; soybean oil 4; salts[‡] 4. The vitamins[§] added per 100 g of diet were: thiamine HCl 1 mg; riboflavin 1 mg; pyridoxine HCl 1 mg; calcium pantothenate 3 mg; niacin 5 mg; choline chloride 100 mg; inositol 100 mg; folic acid .6 mg; biotin .02 mg; vit B_{12} .003 mg; ascorbic acid 50 mg; alpha tocopherol 2 mg; 2-methyl-1, 4-naphthoquinone 1 mg; vit. A 2000 I.U. and vit D 285 I.U. In some diets gum arabic was substituted for celluflour, but since it had only a slight effect on the acid-base balance, data for gum arabic and celluflour diets were combined. The basal diet contained about 0.4% of phosphorus and 0.9% of calcium. The salt mixture of Richardson and Hogan (6) was used at a level of 5% to give a phosphorus content of 0.9% and $NaH_2PO_4 \cdot H_2O$ was added to give a phosphorus content of 1.7%. Magnesium was added as MgO, potassium and sodium as acetates, and calcium as the carbonate. All additions were made at the expense of sucrose. Diets were analyzed for calcium, magnesium, sodium, potassium and phosphorus, and are reported to the nearest tenth of a percent. Chloride and sulphur contents of diet were calculated from the composition of the salts mixture and the protein. For the purpose of calculating the acid-base balance of diets the method of Shohl and Sato(7) was employed except the results are recorded as milliequivalents of excess cations per 100 g. For growth studies guinea pigs weighing 175 to 225 g were fed *ad libitum* for 8 weeks. Most of blood and urine studies were made at the end of this period, but in the case of more severe rations, very few animals survived 8 weeks. For blood studies on these rations older animals were used and an adjustment period of 2 to 4 weeks was allowed before samples were taken. Blood for carbon dioxide capacity was taken by cardiac puncture while animals were under ether anesthesia and the determination was made on plasma by the manometric method of Van Slyke(8). Urine samples for both pH and ammonia determinations were removed directly from the bladder by catherization of anesthetized animals. The pH was determined immediately by the glass electrode and urinary ammonia determined on 5 ml of pooled sample by the method of Van Slyke and Cullen(9). Blood for determination of inorganic phosphorus was collected by clipping a clean toe nail and a 0.1 ml sample was immediately hemolyzed in water. Within 30 minutes the sample was deproteinized with

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[‡] The salts mixture described by Hubbell *et al.*,(5) to which was added 2.5 g of $MnSO_4 \cdot 4H_2O$/100 g.

[§] Folic acid was supplied by Lederle Laboratories, Pearl River, N. Y., biotin by Hoffmann-LaRoche, Nutley, N. J., and other crystalline vitamins by Merck and Co., Rahway, N. J.

TABLE I. Effect of Mineral Composition of Diet on Rate of Gain, Blood Phosphorus, Urine pH and Plasma Carbon Dioxide Capacity.

Ration No.	Variable constituents*				Excess cations per 100 g, mEq	Daily gain (8 wk),† g	Whole blood P, mg/100 ml	Urine pH	Plasma CO_2 cap., vol %
	P	Na	K	Mg					
	%								
3884	.4	.1	.4	.1	21	3.2 (11)‡	6.0 (11)	7.3 (17)	50 (10)
3480	.9	.3	.4	.1	1	1.6 (14)	8.1 (23)	5.9 (11)	50 (3)
3475	1.7	.8	.4	.1	–20	.5 (6)	8.6 (36)	5.6 (13)	34 (10)
3479	.4	.1	1.5	.4	74	4.7 (18)	5.3 (20)	8.2 (20)	48 (14)
3515	.9	.3	1.5	.4	53	3.5 (8)	5.3 (28)	7.8 (8)	50 (6)
3477	1.7	.8	1.5	.4	32	3.8 (16)	7.5 (55)	7.2 (25)	46 (21)
16§	.9	.3	1.8	.25	—§	5.2 (24)	5.0 (58)	8.1 (11)	49 (18)

* Calcium content of all diets was 0.9% except No. 16 which contained 1.5%.

† Avg of mean daily gains of males and females.

‡ No. of animals on which avg is based is shown in parentheses.

§ Ration 16 was a stock diet composed of natural feedstuffs. Analysis was incomplete.

trichloroacetic acid and the method of King (10) was followed.

Results. The effects of different levels (0.4, 0.9 and 1.7%) of dietary phosphorus on rate of gain, blood inorganic phosphorus, urine pH and plasma carbon dioxide capacity are shown in Table I. The levels of calcium (0.9%), potassium (0.4%) and magnesium (0.1%) are those commonly used in purified diets for rats and chicks and these animals thrive on such diets even with calcium to phosphorus ratios as close as 1:1. However, in the guinea pig rate of growth was subnormal even at a low level of phosphorus (3884) unless the diet was supplemented with potassium and magnesium (3479). With higher levels of phosphorus rate of gain became progressively less until at a level of 1.7% most animals succumbed and survivors grew extremely slowly. Blood phosphorus rose to an average of 8.6 mg%, urine pH dropped to 5.6, and plasma carbon dioxide capacity dropped to 34 vol. %. These values should be compared with those obtained on animals fed the stock diet (16) composed of commonly used foodstuffs. The data show a positive correlation between rate of growth and urine pH and a negative correlation between rate of growth and blood inorganic phosphorus. It is obvious that the high phosphorus diet (3475) is an acid diet not only from the calculated deficit of cations, but also from its effect on urine pH and plasma carbon dioxide capacity. Addition of potassium and magnesium to the diets at all levels of phosphorus markedly improved growth, lowered blood phosphorus and raised urine pH. Addition of potassium and magnesium to a ration which contained 0.9% of phosphorus (3515), gave a nearly normal blood and urine picture. Addition of these cations to the excessively high phosphorus ration (3477) improved the rate of growth and raised urine pH, but did not maintain normal blood phosphorus and urine pH values. Thus, the beneficial effect of high levels of potassium and magnesium in the diet of the guinea pigs is due partly, but not wholly, to the maintenance of a positive base balance and a normal level of blood phosphorus.

Of the indices studied the urine pH seemed

TABLE II. Effect of Various Cations on the Urine pH of Guinea Pigs Fed a High Phosphorus Diet.

Ration No.	Additions			Urine pH	Daily gain* (8 wk)
	Cation	%	mEq per 100 g		
3475	None	—	—	5.6 (13)†	.5 (6)
3895	K‡	1.1	28	6.5 (9)	.9 (10)
4120	K‡	2.2	56	7.4 (8)	2.0 (4)
3780	Na§	.6	26	6.7 (6)	1.7 (6)
3889	Na‡	.5	22	6.3 (4)	.7 (14)
3897	Na + Mg	1.0 .3	44 25	7.6 (6)	3.8 (7)
3477	K + Mg	1.1 .3	28 25	7.2 (25)	3.8 (16)
3894	Mg	.3	25	6.4 (9)	2.1 (9)
4119	Mg	.6	50	7.0 (11)	2.1 (4)
3904	Ca	1.6	80	6.8 (9)	2.0 (8)

* Avg of mean daily gains of males and females.

† No. of animals shown in parentheses.

‡ Sodium and potassium were supplied as acetate.

§ Sodium was supplied as Na_2HPO_4 which replaced $Na_2HPO_4 \cdot H_2O$ in the diet.

TABLE III. Urinary Ammonia Excretion in the Guinea Pig.

Ration No.	Excess cations per 100 g, mEq	Urinary NH_3-N, mg/100 ml	Urine pH
3884	21	1.1 (2)[*]	7.3 (17)
3479	74	.9 (2)	8.2 (20)
3475	–21	1.1 (3)	5.6 (13)
16	—	1.4 (3)	8.1 (11)
Human urine	—	60.0 (2)[†]	6.5 (2)

[*] No. in parenthesis indicates No. of determinations. Urine from several animals was pooled for a determination.

[†] These values were determined on freshly voided samples and hence do not represent a 24 hr specimen, but they are in the range commonly given for normal human urine.

to be the most sensitive to slight changes in acid-base balance. Hence, it was used to study the relative value of various cations in counteracting the injurious effects of a high phosphorus diet. Results of the growth studies and urine pH determinations are shown in Table II. Addition of each of the cations studied, potassium, sodium, magnesium and calcium, improved rate of growth and raised the urine pH. Within limits, the cations were of about equal efficacy and the effect was in proportion to the quantity added. Although potassium is more abundant than sodium in the natural diet of herbivorous animals, as a supplement to the basal diet, sodium appeared as effective as potassium either alone or in combination with magnesium. On an equivalent basis none of the cations was as effective alone as in combination. Calcium ion was quite effective in raising urine pH because in the guinea pig a relatively high proportion of the calcium is excreted by way of the kidneys (unpublished data).

The guinea pig like the rabbit(11) is more sensitive to an acid diet than most animals chiefly because it does not use ammonia to neutralize excess acids excreted by way of the kidney. In Table III are shown results of ammonia nitrogen analyses of fresh urine samples. These values on guinea pig urine agree very well with those reported for rabbit urine(12) but are of the order of 2% of the ammonia commonly found in human urine. Urine from animals fed a high phosphorus diet had a low pH but the amount of ammonia was approximately the same as that found in urine of a high pH. Thus, consumption of an acid diet caused a change from basic to acid phosphates in the urine, but it did not stimulate the excretion of urinary ammonia. Guinea pigs that consume a stock colony diet normally excrete an alkaline urine which is quite turbid. Qualitative analysis of the insoluble portion indicates that it is composed chiefly of calcium and magnesium phosphate and microscopic examination shows that phosphates are held in a non-crystalline, colloidal state. The urine excreted when animals consume a high phosphorus diet, is not turbid but is clear in appearance.

Since there is a negligible amount of ammonia excreted in the urine of the guinea pig, the shift to an acid urine appears to be the chief mechanism for conserving fixed bases. This mechanism was effective in maintaining a normal plasma carbon dioxide capacity when the level of dietary phosphorus was 0.9%, but at a level of 1.7% of phosphorus there was marked drop in carbon dioxide capacity. It seems possible that high blood phosphorus and the incidence of soft tissue calcification observed when animals consume a high phosphorus diet that is relatively low in potassium and magnesium are due in part to the inability of the guinea pig to excrete phosphorus normally under the conditions of a low base reserve.

Summary. Injurious effects of high phosphorus diets for the guinea pig, which include poor growth, metastatic calcification, and death, are due in part to the fact that this animal does not tolerate an acid diet. This has been demonstrated by the marked drop in urine pH, a decrease in plasma carbon dioxide capacity and an increase in blood inorganic phosphorus. Addition of various cations produced a more alkaline urine, but a combination of sodium or potassium and magnesium proved most beneficial. The guinea pig is highly sensitive to an acid diet because a negligible amount of ammonia is excreted by way of the kidneys.

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A Histamine-Like Component of Commercial Pitressin.* (22219)

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Several investigators have assigned an important role to pitressin as a releasing agent for ACTH. Thus, Mirsky, Stein and Paulisch(1), suggested that the antidiuretic hormone of the posterior pituitary (ADH, Vasopressin) may serve as a mechanism for ACTH release under stress. McCann and Brobeck (2), also implicated ADH as a factor for release of corticotropin. They destroyed the supraoptico-hypophyseal tract thereby inhibiting the usual output of ACTH in response to stress. However, when animals bearing hypothalamic lesions were injected with commercial pitressin, ACTH was released. Guillemin and Hearn(3), employing rat pituitaries and a tissue culture technic, demonstrated that pitressin releases ACTH from the adenohypophysis *in vitro,* whereas highly purified arginine-vasopressin obtained from du Vigneaud was without effect. The ACTH releasing activity of pitressin was attributed to an unknown contaminant probably originating in the hypothalamus. Saffran, Schally and Benfey(4), also reported that vasopressin contained a corticotropin-releasing factor as an impurity which they were able to separate from vasopressin by paper chromatography.

In view of reports(5-7) that extracts prepared from posterior pituitary powder and fresh glands contain histamine, it was considered possible that minute amounts of the amine bound to the polypeptide were carried through the extraction procedure for commercial pitressin and persisted in the finished product. To anticipate, it can be stated that commercial pitressin contains a component of unknown nature which exhibits some of the physiological characteristics of histamine and which for brevity we shall hereafter refer to as "histamine-like."

Methods. Pitressin† (Vasopressin Injection, 20 units/cc, Parke, Davis and Co.) was extracted with strong acid at boiling temperature. The material was pooled in batches containing 3200 units, in approximately 160 cc, and sufficient concentrated HCl added to make an 18% solution. The fluid was divided among 3 flasks equipped with water cooled reflux condensers and the pitressin boiled vigorously for 1 hour. The commercial material is preserved with 0.5% chlorobutanol which readily crystallizes on the condenser and was removed. After 1 hour the water condensers were replaced by reflux air con-

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† We are indebted to Dr. D. A. McGinty of Parke, Davis and Co., for 3000 units of pitressin.

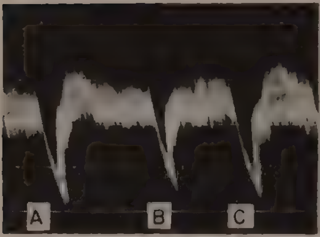

Fig. 1

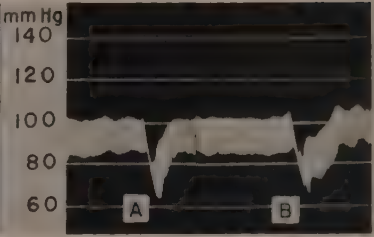

Fig. 2

FIG. 1. Cat, 2.3 kg, atropinized, etherized, carotid blood pressure. A: Pitressin extract 2 cc i.v. (625 units/kg). B: Histamine base 1.81 γ/kg i.v. C: Histamine base 3.62 γ/kg i.v. One cc of pitressin extract is equivalent to 4.17 γ/cc histamine base.

FIG. 2. Cat, 1.9 kg, atropinized, etherized, carotid blood pressure. A: Histamine base 0.3 γ/kg i.v. B: Oxytocin extract 1.5 cc i.v. (625 units/kg). Slight depressor effect. One cc of oxytocin extract is equivalent to 0.38 γ/cc histamine base compared to pitressin extract = 4.17 γ/cc of histamine base.

densers and the boiling continued for an additional 30 minutes or until the fluid was reduced to 10-15 cc. The contents of the flasks were then combined and the extract taken to dryness under reduced pressure at 40°C. The residue was washed 3 times with absolute ethanol which was removed *in vacuo*. Water was added to the desired concentration and the pH of the solution raised to that of commercial pitressin, *i.e.,* pH 3. A fine precipitate forms which is removed by centrifugation. Crude extracts prepared in the manner described retained no trace of pressor activity when tested on the blood pressure of the dog and cat. The histamine-like substance in the extracts was tested by its effect on smooth muscle of the guinea pig ileum and blood pressure of the etherized, atropinized cat. Segments of the lower ileum of fasted young guinea pigs were suspended in a 10 cc chamber containing warm, oxygenated, magnesium-free Tyrode's solution to which atropine had been added in the concentration 0.01 γ/cc. The active agent in all extracts was determined as quantitatively equivalent to histamine base and pyribenzamine was employed for inhibiting contractions of the ileum and for preventing the decline in blood pressure of the cat following extract or histamine administration.

Results. The pressor action of pitressin is abolished by boiling for 30-40 minutes in 1% HCl but a pronounced histamine-like effect is exhibited by concentrated extracts only after prolonged boiling in much stronger acid. Fig. 1 shows the effect of a concentrated extract representing 762.5 pitressin units/cc, or a 38-fold concentrate of acid-boiled commercial material. Strong depressor activity occurred which resulted in a 40 mm Hg fall in blood pressure when 2 cc were given by vein to the etherized, atropinized cat. The extract was estimated to contain activity equivalent to 4.17 γ/cc of histamine base. Oxytocin, prepared in the same manner as pitressin extract, concentrated to the same degree and administered in the same dosage to the atropinized, etherized cat, gave minor depressor effects (Fig. 2). The pitocin contained activity approximating 0.38 γ/cc of histamine base, hence possessed less than one-tenth the activity of pitressin. Apparently the histamine-like substance in the posterior lobe is chiefly confined to the pitressin fraction.

Concentrated pitressin extracts also induced typical histamine-like responses when tested on the guinea pig ileum (Fig. 3). The extracts contained 125 original pitressin units/cc, and the histamine base equivalent was approximately 0.3 γ/cc. Pyribenzamine abolished the gut response to both extract and histamine (Fig. 3 C, D).

McCann and Brobeck(2), were unable to deplete the adrenal ascorbic acid of hypophysectomized rats by intravenous injection of pitressin. We also obtained negative results in similar experiments. Extract concentrates representing 803 units/cc, and containing significant amounts of histamine-like activity in tests on the cat's blood pressure (35 mm Hg

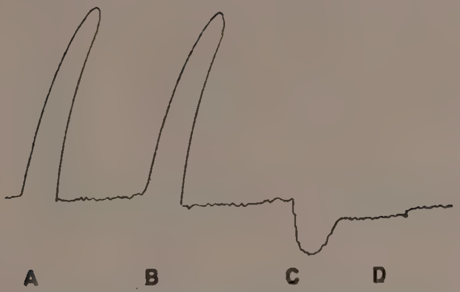

FIG. 3. Isolated ileum of guinea pig. Doses given per cc of bath fluid. A: Histamine base 0.015 γ/cc. B: Pitressin extract 0.50 cc. One cc of extract equivalent to 0.3 γ histamine base. Between B and C pyribenzamine given 0.5 γ/cc. C: Pitressin extract 0.5 cc. D: Histamine base 0.015 γ/cc.

fall), failed to produce a decline in adrenal ascorbic acid of 8 hypophysectomized rats. The extract was injected in 0.4 cc doses by vein. We are indebted to Dr. H. Megel of the Princeton Laboratories, Inc., for making the Sayers test for us.[‡]

Discussion. Abel and Kubota(5) reported histamine present in posterior pituitary powders and extracts. Subsequent investigators failed to detect the amine in either powders (8) or fresh glands(9). Recently, Harris *et al.*(7) were able to extract histamine from brain, hypothalamus, median eminence and both anterior and posterior pituitary tissue. The present writers have found a histamine-like substance present in extracts of acetone dried powders of the posterior lobe. Four grams of such powder when subjected to the complete extraction procedure of Code and Ing(10) for determining the presence of histamine in blood plasma and cells, and highly concentrated, contained activity approximating that of 4.19 γ/cc, of histamine base when assayed against the standard and using the guinea pig ileum and blood pressure of the cat as test objects.

We were not successful in removing the active agent from pitressin or acetone dried powder by aqueous or dilute HCl extraction. It required strong acid and 90 minutes of boiling temperature to accomplish its removal. Apparently the histamine-like component of pitressin is bound to the pressor polypeptide and is released, or at any rate rendered capable of inducing physiological reactions characteristic of histamine, by some degree of acid hydrolysis. Pyribenzamine readily inhibited the response of the gut to extract and to pure histamine dihydrochloride, but it did not entirely eliminate the depressor effect of 2 cc of concentrated extract on the blood pressure of the atropinized cat. Frandsen(11) reported that the active histamine-like substance found in ultrafiltrates of dog serum differed from pure histamine since it is not destroyed by added histaminase nor attacked by histaminase of serum. The exact nature of the histamine-like component in pitressin is unknown but if it is histamine it does not appear to be present as the free amine. The writers tentatively regard it as probably histamine combined with an amino acid or peptide, but pending its isolation in pure form this is merely speculation. Certain facts seem to favor the idea and may be summarized as follows: In tests on the guinea pig ileum the behavior of the active substance in the extracts cannot be distinguished from that of histamine and like the amine its action is abolished by pyribenzamine. When highly concentrated it exerts strong depressor effects on blood pressure of atropinized, etherized dogs and cats and the slope of the pressure falls and subsequent recovery is identical with that elicited by histamine. The effect of the extract upon ileum and blood pressure can be quantitated using histamine base as the standard. Similar extracts of oxytocin which differs from pitressin by two amino acids, elicit small depressor responses in atropinized cats, but they are less than one-tenth the magnitude of those induced by pitressin. Dosage in both cases was 625 units/kg. Extracts of 4 crystalline proteins and of each of the amino acids in vasopressin also gave negative results. Investigators(2-4) have shown that oxytocin, unlike pitressin, does not release ACTH. Histamine as the dipicrate has been recovered from posterior lobe tissue (5,6) and a histamine-like substance has been reported present in this gland and also in neighboring hypothalamic structures(7). None of these data furnish conclusive evidence that a histamine complex of some sort is the active component of pitressin extracts but they are suggestive, and it seems not unreasonable to make such an assumption as a working hypothesis.

[‡] Recent experiments show the extract to be active in releasing ACTH from halved anterior pituitaries incubated in the Warburg apparatus using the Saffran *et al.* technic. Minute amounts of the incubation fluid given i. v. to 24 hypophysectomized rats induced a significant fall in adrenal ascorbic acid of 120 mg % in the Sayers test. Incubation fluid from the control half of the same glands gave negative results.

Summary. Commercial pitressin contains minute amounts of a histamine-like component apparently bound to the polypeptide but which can be concentrated and rendered capable of inducing physiological responses characteristic of histamine when pitressin is subjected to strong acid hydrolysis and the pressor activity destroyed. Extracts of commercial oxytocin contained but one-tenth the vasodepressor activity of pitressin extracts.

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Effects of Glucagon on Plasma Potassium.* (22220)

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Glucagon(1) has a selective action on liver in which the activity of phosphorylase is increased and this action may be the basis for increased hepatic glycogenolysis and hyperglycemia(2). The effects of glucagon mimic epinephrine action only in the liver. Glucagon has no glycogenolytic action on muscle, no cardiovascular effect(3), and no effect on hepatic vessels(4). These properties make glucagon a useful pharmacologic tool for studying the accompaniments of hepatic glycogenolysis. Our aim was to determine whether hepatic glycogenolysis was necessarily associated with increased blood potassium.

Although epinephrine hyperkalemia has been related to hepatic glycogenolysis, several facts appear inconsistent with this simple relationship. Subcutaneous epinephrine produces hyperglycemia without an associated hyperkalemia. Intravenous epinephrine produces hyperkalemia which persists for only 3 to 5 minutes(5) and hyperglycemia which continues for one-half to one hour. The relative potencies of sympathomimetic amines for the hyperkalemic effect are markedly different from their relative potencies for hyperglycemia(6,7). Dibenamine is effective in suppressing epinephrine hyperkalemia(7), but it is extremely weak as an antagonist to epinephrine hyperglycemia(8). Epinephrine hyperkalemia occurs in animals treated with phloridzin in order to deplete liver glycogen (9). Constrictor agents with weak hyperglycemic activity produce hyperkalemia(5, 10). These discrepancies were the basis for

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investigating blood potassium effects of glucagon, an agent which has hyperglycemic activity related in detail to the action of epinephrine but which has no vascular effect.

Methods. Cats, 1.85 to 3.0 kg were anesthetized with an intraperitoneal injection of 0.88 ml per kg of Dial with Urethane (each ml contains 0.1 g diallylbarbituric acid, 0.4 g urethane, and 0.4 g monoethylurea). Arterial blood samples and mean arterial pressure were obtained from a polyethylene catheter introduced into the right carotid artery and passed down into the region of the aorta. Arterial samples of approximately 3 ml were taken into heparinized syringes. Blood samples were cooled in an ice bath and the plasma separated in a refrigerated centrifuge. Control samples were taken 15 and 5 minutes before the injection. Plasma glucose was determined with the method of Nelson(11) and Somogyi(12) and plasma potassium was determined with a Beckman DU Spectrophotometer with flame attachment. Drugs were injected into a jugular or femoral vein. Glucagon[‡] was an amorphous powder (Lot No. 208-158B-197) which contained approximately 50% glucagon and less than 0.05 unit of insulin per mg. In comparable experiments synthetic l-epinephrine bitartrate was used.

Results. Our data indicate that glucagon has an effect on plasma potassium similar to the effect of epinephrine. There is a marked, transient hyperkalemia followed by a slight hypokalemia of much longer duration. Glucagon induced an hyperglycemia which reached its peak much later than the hyperkalemia and hyperglycemia was present during the much longer period of hypokalemia.

The effects of glucagon (50 μg/kg i.v.) on the potassium and glucose of the arterial blood plasma are shown in Fig. 1. This is a graph of the averages of 5 experiments of similar duration. In a total of 8 experiments the average rise in plasma potassium one minute after the glucagon was administered was 2.20 mEq./l. (range 1.28-3.50) from an average control level 3.63 mEq./l. (range 3.14-4.31). The control plasma potassium and the effect of glucagon on plasma potassium were not proportional to the control plasma glucose nor to the rise in glucose. Both fasted and nonfasted cats were used. Control plasma glucose levels were from 73 to 198 mg%.

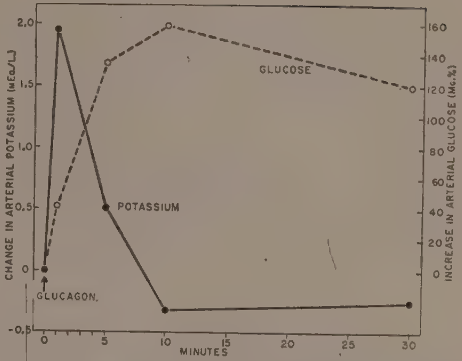

FIG. 1. Effects of glucagon (50 μg/kg) on arterial plasma potassium and glucose concentrations. Points indicate averages of 5 experiments in cats anesthetized with Dial.

Similar hyperkalemic and hyperglycemic effects were obtained with epinephrine 10 μg/kg i.v. Epinephrine produced its characteristic pressor action, whereas glucagon had no effect on the blood pressure, on the heart rate, or on the electrocardiogram. In control experiments there were no changes in arterial potassium one minute after the intravenous administration of saline or of glucagon-free insulin[‡] (50 μg/kg).

With the exception of the hyperkalemic effect of glucagon, which does not appear to have been observed previously, our results confirm previous data in the literature. The subsequent hypokalemia would be expected as a consequence of the hyperglycemia which can induce a discharge of insulin and a greater tissue storage of potassium.

Discussion. It appears that glucagon, as well as epinephrine, produces a transient rise in arterial plasma potassium (presumably derived from the liver) during the activation of hepatic glycogenolysis. Since epinephrine and glucagon have the same effect on liver glycogenolysis, but only epinephrine has a vasoconstrictor action(13), it appears reasonable to conclude that there may be some relation of the hyperkalemic effects of these agents to hepatic glycogenolysis rather than to any vascular action. There are still several facts which do not appear to be consistent with this simple relationship. Some of these have been noted in the introduction. Although hyperkalemia and hyperglycemia appear to be associated responses, the former is completed in a few minutes while the latter persists. More related in time are the duration of hyperkalemia and the rising phase of hyperglycemia. The possibility exists that potassium loss from the liver occurs during the activation of phosphorylase, a process which occurs in less than four minutes(14), and which, under present experimental conditions, may be restored to the resting state in less than 10 minutes.

[‡] Glucagon and glucagon-free insulin were obtained through the kind cooperation of Dr. W. R. Kirtley, Lilly Research Laboratories, Indianapolis, Ind.

The repeated release of potassium from the liver was demonstrated by D'Silva(9) at intervals of 3 minutes. These experiments involved the repeated injection of epinephrine at 3 minute intervals and the withdrawal of arterial samples one minute after each injection. Since the liver has completed its release of potassium and may be reabsorbing potassium 3 minutes after an injection of epinephrine, D'Silva's data do not necessarily represent a continuous release of potassium.

The simultaneous loss of potassium and glycogen from the liver would appear to be in accord with Fenn's concept that there is a relation between the storage and loss of glycogen and potassium in tissues(15). However, the loss of potassium is rapid, but transient, and there is replenishment of hepatic potassium during the extended period of hyperglycemia. More information on hepatic phosphorylase activity may explain this curious situation.

Summary. In the cat, glucagon has an effect on arterial plasma potassium like that of epinephrine. It produces a transient, marked hyperkalemia followed by a much longer, but milder hypokalemia. Thus, glucagon is similar to epinephrine in that it elevates blood potassium and glucose and dissimilar in that it has no cardiovascular effects.

We are indebted to Dr. A. J. Plummer, Ciba Pharmaceutical Products, Summit, N. J., for a generous supply of Dial® with Urethane.

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Toxicity of Penicillin for the Syrian Hamster. (22221)

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Observation of the unique toxicity of penicillin to guinea pigs as compared to mice and rabbits was first made by Hamre *et al.*(1) and has since been amply confirmed(2-8). Except for generalized state of vasodilatation, very few pathological changes have been noted to account for death of the animals. The cause of this toxic effect has been variously ascribed to:—acute necrosis involving the adrenal gland, particularly of the cortex (4); allergy to penicillin(6); possible deficiency state with effects very similar to those produced by X-rays(7); and finally to change induced by penicillin in predominantly Gram positive intestinal flora of guinea pigs thereby permitting overgrowth of non-sensitive Gram negative bacteria and production and resorption of toxins of the latter(8).

Existence of this unusual sensitivity to penicillin has not been reported in other animals. During experiments on Syrian hamsters treated with penicillin, it was first noticed that mortality rate of treated group consistently exceeded that of untreated controls. Because of observations in guinea pigs and of their widespread use in research, we investigated the possible toxic effect of penicillin upon hamsters.

Methods. Syrian hamsters, of either sex, weighing 50-60 g were employed in groups of 10. A single injection of crystalline penicillin G potassium (Bristol) was administered to each hamster in dose and route indicated in Table I. Volume injected subcutaneously was 0.25 ml and intraperitoneally 1 ml. All animals were kept on normal diet and observed 21 days. Symptoms were noted, total mortality and days of death were recorded for each group. Because of reported resistance of mice and susceptibility of guinea pigs to penicillin, these were included for comparison. Two groups of 10 Swiss mice, weighing 15-20 g and 2 groups of 10 guinea pigs, weighing approximately 200 g received 100000 units of crystalline penicillin G potassium. One group of each species was injected subcutaneously and the other intraperitoneally in 0.25 ml and 1 ml amounts, respectively. In addition, groups of each species received the same volume of physiological saline by same route as those receiving penicillin.

TABLE I. Toxicity to Syrian Hamster and Mortality Time of One Injection of Variable Doses of Crystalline Penicillin G Potassium Administered Subcutaneously and Intraperitoneally.

	Subcutaneous		Intraperitoneal	
Penicillin (units)	Mortality	Days of death (No.–day)	Mortality	Days of death (No.–day)
×1000				
1	1/10*	1–7	0/10	—
5	”	1–3	2/10	1–2; 1–3
10	3/10	1–3; 2–4	4/10	2–2; 2–3
25	”	3–3	5/10	3–3; 2–4
50	5/10	1–3; 2–4; 1–8; 1–12	9/10	3–3; 4–4; 1–5; 1–6
100	9/10	4–4; 4–5; 1–7	10/10	3–4; 6–5; 1–6

* Numerator = No. died. Denominator = Total No. animals injected.

Results. No deaths were observed in control animals in the 3 animal species under consideration that received physiological saline either subcutaneously or intraperitoneally. From Table I, it may be noted that a single injection of penicillin starting with 1000 units subcutaneously and 5000 units intraperitoneally is toxic for some hamsters. Increasing the dose by either route results in progressively higher mortality until a 90-100% rate is observed with 100000 units. Intraperitoneal administration proved more toxic than did subcutaneous, as evidenced by consistently higher mortality rate in animals receiving the antibiotic by former route. The peak mortality period for varying doses of penicillin is also illustrated in Table I. No deaths occur on 1st day, only 3 out of 52 deaths are found on 2nd day and vast majority of deaths takes place on 3rd, 4th and 5th days following injection. Few deaths supervene after this time. It is of interest to

TABLE II. Comparative Susceptibility of Swiss Mice, Guinea Pigs and Syrian Hamsters to One Injection of 100000 Units of Crystalline Penicillin G Potassium.

	Subcutaneous		Intraperitoneal	
Animal	Mortality	Days of death (No.–day)	Mortality	Days of death (No.–day)
Mice	0/10	—	0/10	—
Guinea pigs	10/10	1–3; 3–4; 3–5; 2–6; 1–7	8/10	1–3; 2–4; 4–6; 1–7
Hamsters	9/10	4–4; 4–5; 1–7	10/10	3–4; 6–5; 1–6

note that increased dosage of penicillin produces higher mortality rate but no shortening of survival time. On the contrary, with higher doses, peak mortality is delayed a day or two rather than accelerated as compared with lower amounts. Thus peak mortality day with 25000 units or less is the 3rd, with 50000 units, the 4th, and with 100000 units the 5th day. The picture presented by the hamsters was similar to that previously described for guinea pigs. Food and water intake was diminished, hair became ruffled, animals were lethargic and huddled together in a shrunken posture.

By contrast, as seen in Table II, Swiss mice weighing only 15-20 g readily tolerate a single injection of 100000 units of penicillin administered either subcutaneously or intraperitoneally. They remain alive and active throughout the 21-day observation period. On the other hand, the same dose of antibiotic causes death in 80-100% of guinea pigs and in 90-100% of hamsters similarly treated, depending upon route of administration. The mortality pattern in hamsters closely parallels that in guinea pigs as toxicity of penicillin for both is of the delayed type. Thus in both species no deaths are noted before the 3rd day or after 7th day following injection.

In the light of our findings, reevaluation of experimental data concerned with Syrian hamsters either treated with penicillin or injected with material sterilized with penicillin would seem to be indicated. Although amounts less than that reported in this study as capable of producing toxic effects in hamsters might have been employed, the cumulative effect of multiple small injections might well produce the same result and render observed experimental data inaccurate.

Summary. A single injection of crystalline penicillin G potassium administered subcutaneously or intraperitoneally is toxic for hamsters in proportion to amount administered. Toxicity is of the delayed type, the vast majority of deaths occurring on 3rd, 4th and 5th days following injection. This parallels the reported experience with guinea pigs and is in contrast to Swiss mice who easily tolerate very large doses of this antibiotic. In the light of these findings, reevaluation of experimental data concerned with Syrian hamsters either treated with or injected with material sterilized with penicillin would seem to be indicated.

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A Serum Peptidase and Its Possible Role in Protease Inhibition. (22222)

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The presence of a number of peptidases in serum has been detected by the use of synthetic substrates such as leucyl-glycyl-glycine, glycyl-glycyl-glycine, glycyl-leucine, etc. The function of serum peptidases, however, is still largely unknown. The method described by Gomori(1,2), using a chromogenic substrate, is sensitive and simple enough to lend itself easily to physiological and clinical studies. The present paper reports the results of investigations carried out with Gomori's method. These results point to the possibility that part of the well-known antiproteolytic activity of serum is due to enzymatic destruction of proteases.

Methods. Determination of enzyme activity is based on the color reaction between beta-naphthylamine and certain diazotized dyes. When glycyl-beta-naphthylamine is hydrolyzed, the amine set free gives a purple colored compound in combination with the dye. Intensity of color, measured spectrophotometrically, indicates degree of hydrolysis of substrate. *Reagents.* 1. Glycyl-beta-naphthylamine (GBNA) was prepared according to Gomori(2) and used at concentration of 0.0002 M (sample used was prepared and kindly supplied by A. J. Merritt, U. S. Vitamin Corp.). 2. Veronal buffer, pH 7.4, 0.15 M in physiological saline. 3. Acetate buffer, pH 4.2, 1 M, containing 10% Tween 20. 4. Red B Salt (Matheson, Coleman and Bell) in 0.25% solution, to be prepared shortly before use. *Procedure.* The enzyme solution is made up to 6 ml with veronal buffer and placed in water bath at 37.5°C. The reaction is started by addition of 2 ml of GBNA solution. After required interval, usually 30 min., the reaction is stopped by adding 1 ml of Tween-acetate buffer solution. The purpose of Tween 20 is to solubilize the colored compound formed when 1 ml of dye is added to the reaction mixture. Color develops rapidly and remains stable for over an hour. Test solutions are read in Coleman Universal spectrophotometer at 550 mμ against blank containing all reagents except the enzyme. *Color reaction* has a high sensitivity and may be made even more sensitive by reducing volume of reagents. The beta-naphthylamine-dye compound has a molar extinction coefficient of 43800 at 550 mμ. With this procedure 2.5% hydrolysis can still be measured (0.01 μM in final volume of 10 ml). Results of experiments are expressed in terms of μM of substrate hydrolyzed. *Inhibition of protease* by serum was measured by means of method described previously(3). Blood samples were collected by venous or heart puncture and allowed to clot at 37.5°C. Unless otherwise stated, the serum was used shortly after separation. When kept for longer periods, serum was frozen to –20°C. Tissue extracts were prepared according to method described by Fruton(4) and extracts were kept frozen.

Results. Serum samples from laboratory animals and healthy human subjects were tested for peptidase activity on GBNA. Table I shows mean values of GBNA hydrolysis by sera from different species at stated concentrations. Although enzyme activity varied from one species to another it was remarkably constant within the same species.

Kinetic studies were carried out with guinea pig serum because of its high activity. They showed that rate of GBNA hydrolysis decreased as the reaction proceeded (Fig. 1).

TABLE I. Hydrolysis of GBNA by Sera from Different Species.

Species	Conc., ml	μM hydrolyzed in 30 min.	± S.E.	No.
Guinea pig	.1	.089	.004	24
Human	.3	.024	.005	4
Mouse	.3	.020	.005	3*
Rabbit	.5	.062	.008	6
Dog	.5	.032	.005	4
Rat	.5	.028	.002	12

* 3 pooled samples from 4 mice each.

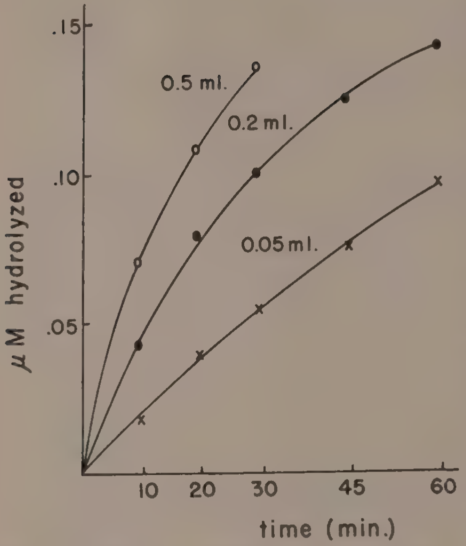

FIG. 1. Hydrolysis of GBNA by guinea pig serum. Abscissa: duration of the reaction (min.); ordinate: μM of substrate hydrolyzed by the amounts of serum indicated.

It was found also, as shown in Fig. 2, that hydrolytic activity was not linearly related to enzyme concentration but approximated more closely log [E]. Rate of hydrolysis, however, was proportional to substrate concentration within limits of experiment. The reaction is not inhibited by its end-products: neither glycine nor beta-naphthylamine have any inhibitory action on the enzyme at the concentrations at which they are released during the reaction.

Hydrolysis of GBNA by guinea-pig serum was inhibited by cyanide and ethylenediamine-tetra-acetic acid (EDTA). This suggested that the enzyme action requires some metallic ion. None of the ions tried (Ca, Mg, Mn, Co, Ni, Cu, Zn), however, activated hydrolysis or reversed action of the chelating agent. Most of the metals tried inhibited GBNA hydrolysis, some of them (Cu, Zn) at very low concentrations.

Enzyme action was also inhibited by a number of amines (methylamine, tryptamine, epinephrine, norepinephrine, histamine, 5-hydroxy-tryptamine). The amino-acids corresponding to some of them (histidine, tryptophan) were inactive. Ammonium salts can also inhibit peptidase; quaternary ammonium compounds (choline, acetylcholine), however, had no inhibitory action. The basic protein, protamine, had a comparatively strong inhibitory action; on molar basis approximately 10^{-7} would be required for 50% inhibition.

The GBNA-hydrolyzing enzyme was found in the albumin fraction when serum was diluted 20-fold with distilled water and precipitated at pH 5.4. Heating serum to 56°C for 30 minutes did not significantly decrease the enzyme activity. Dialysis in the cold for 48 hours against saline did not cause loss of activity. Serum which was kept frozen for 24 hours showed consistently higher activity (by 15 to 25%) than fresh serum.

A few experiments with tissue extracts showed that guinea-pig lung and skin were able to hydrolyze GBNA (0.130 μM by 100 mg of skin) while rat tissues were inactive. Human urine showed slight activity (0.016 μM/ml). It appeared that the GBNA-hydrolyzing enzyme had striking similarities with the substances variously designated as antitrypsin, antifibrinolysin, antiplasmin, protease inhibitor, etc. GBNA hydrolysis and plasmin (fibrinolysin) inhibition follow similar reaction kinetics: the rate is highest in the

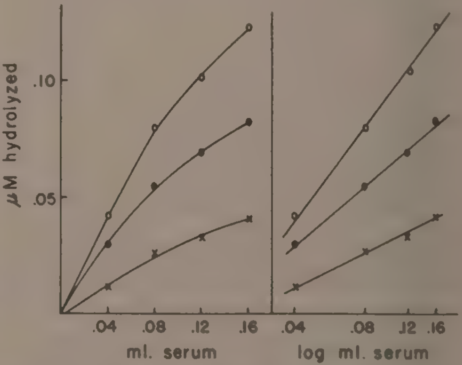

FIG. 2. Hydrolysis of GBNA as a function of enzyme concentration. Ordinate: μM of substrate hydrolyzed in 30 min. at the substrate concentration indicated; abscissa: amount of guinea pig serum added to the substrate (ml) at arithmetic (left) and logarithmic (right) scale.

× = .2 μM GBNA
● = .4 " "
○ .6 " "

TABLE II. Inhibition of GBNA Hydrolysis by Fibrinolysin and Trypsin.

	μM hydrolyzed in 30 min. by	
Inhibitor,* mg	.1 ml guinea pig serum	.2 ml guinea pig serum
0	.085	.110
Plasmin 10	.078	.096
25	.060	.076
50	.040	.045
Trypsin 0.4	.082	.104
1.0	.061	.080
2.0	.037	.046

* Conc. for total volume of 8 ml.

Serum and inhibitor were left in contact for 10 min. before addition of substrate.

first 5 min. and increases linearly with the log of serum concentration(3,5). Both reactions are inhibited by EDTA and inhibition is not reversed by addition of Ca(6). Both systems are inhibited by low concentrations of Cu and protamine(7), by certain amines and ammonia(8). On isoelectric precipitation, both properties of serum are found in the supernatant(9).

One more evidence is supplied by the observation that trypsin (Tryptar, Armour Laboratories) and plasmin (bovine preparation kindly supplied by Dr. E. C. Loomis, Parke, Davis and Co.) inhibit hydrolysis of GBNA by guinea pig serum (Table II). Several other globulin fractions from guinea pig, rabbit and human serum showed no inhibitory action. Plasminogen did not inhibit but inhibitory activity appeared after activation with streptokinase. Kinetic studies of this inhibition are now in progress; they indicate that it is competitive and reversible. Reversibility of the inhibition was substantiated by the following experiment. Guinea pig serum was incubated with plasmin for 30 min. The mixture was then diluted with distilled water and the globulin fraction precipitated out at pH 5.4. Plasmin was in the precipitate while GBNA-peptidase remained in solution. By testing the 2 fractions, it was seen that plasmin was partly destroyed but the peptidase was completely recovered. GBNA and proteases seem to be competing substrates for the same enzyme.

The GBNA-hydrolyzing enzyme, however, is not the only protease-inhibiting factor in serum. There are probably two(10) and possibly three(8) antiproteases in human serum. Table III shows the correlation between plasmin inhibition and GBNA hydrolysis by sera from different species. It is seen that in guinea pigs and possibly rabbits the plasmin-inhibiting action of serum may be due to GBNA-peptidase. In other species, particularly in man, other inhibitors may play a more important role. Bovine serum has no action on GBNA and purified bovine antiplasmin prepared by Loomis(11) also fails to act on this substrate.

The plasmin inhibitor of guinea-pig serum controlled by pituitary and adreno-cortical hormones(3,12) may be identical with GBNA-peptidase. Table III shows that sera from guinea pigs treated with cortisone (5 mg/kg, 4 hours before bleeding) had an increased action on GBNA hydrolysis and plasmin inhibition. The opposite was observed in sera of animals treated for 4 days with 5 mg/kg of pituitary growth hormone (obtained by courtesy of Armour Laboratories).

Discussion. It is uncertain whether the enzyme detected by its action on GBNA is identical or not with one of the peptidases already described in serum. It seems to be closest to the enzyme hydrolyzing glycyl-leucine which, however, is activated by Zn(13). The relationship of GBNA peptidase with metallic ions remains unknown; its activation by Co, observed in human serum(2), was not confirmed in guinea pig serum. It seems that, if it requires any ion at all,—as is suggested by EDTA inhibition,—this has to be firmly

TABLE III. GBNA Hydrolysis and Plasmin Inhibition by Sera from Different Species.

Species	μM GBNA hydrolyzed/ml in 30 min.	Units plasmin inhibited/ml
Mouse	.030	40
Rat	.036	44*
Human	.038	51
Dog	.041	47
Rabbit	.081	36
Normal guinea pig	.143	66*
Cortisone-treated guinea pig	.194	85*
Growth hormone-treated guinea pig	.082	31*

* Results published in a previous study(3).

bound to the protein since it cannot be removed by dialysis.

Inhibitory action of certain biologically important amines (serotonin, histamine) is unlikely to have any physiological significance because they can hardly occur at the required concentrations.

On the basis of present data it cannot be decided whether GBNA-hydrolyzing enzyme is a true peptidase or a proteinase whose specificity is satisfied by the structure of the synthetic substrate. To inactivate trypsin or plasmin, it has to hydrolyze off their active groups. Since the nature and length of these groups is unknown we cannot even speculate on site of action of the enzyme.

The fibrinolytic system and other similar systems present in tissues appear to play a role in certain physiological and pathological processes(14). Rate of protease inhibition is an important factor controlling extent and duration of the processes. If the interpretation of present results is confirmed, they may supply an interesting example of one enzyme controlling the activity of another.

Summary. A peptidase, detectable by its action on chromogenic substrate glycyl-beta-naphthylamine, is present in serum of most species. In guinea pig serum, hydrolysis of GBNA and protease inhibition show a marked parallelism: they have a similar kinetic behavior, they are inhibited by the same agents and are under the influence of the same hormones. Competitive inhibition of peptidase action by plasmin and trypsin suggest that proteases are substrates for peptidase and that antiproteolytic activity of guinea pig serum, is due, partly at least, to enzymatic destruction of proteases. In some other species this mechanism may be less important.

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Observations on a Precipitin Reaction Between Serum of Patients with Rheumatoid Arthritis and a Preparation (Cohn Fraction II) of Human Gamma Globulin.* (22223)

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Since the observation by Rose *et al.*(1) of agglutination, by sera of many patients with rheumatoid arthritis, of sheep red cells sensitized with nonagglutinating quantities of rabbit antisheep cell serum (amboceptor), a number of modifications of this test system have been described. These fall into two types: those using sheep red cells sensitized with amboceptor against patient's serum or its euglobulin fraction and a system described by Heller in 1954(2). In the latter, sheep red cells, previously treated with tannic acid, are coated with pooled human gamma globulin (Cohn Fraction II). These treated cells are agglutinated when exposed to the serum of patients with rheumatoid arthritis.

In numerous clinical studies(3,4), these test systems have shown a high degree of specificity in patients with indisputable evidence of rheumatoid arthritis. That these tests remain positive when the overt inflammatory reactions have been suppressed by steroid therapy has suggested to us that the circulating factor or factors (rheumatoid factor, R.F.) responsible for these serological reactions may be closely concerned with the mechanisms of this disease. While studying the mechanisms involved in the Heller FII test, it appeared likely that there existed a specificity of interaction between some substance in human pooled gamma globulin and the rheumatoid factor (R.F.), the interaction product of these two on sheep red cells producing visible agglutination. Although the R.F. in the patient's serum has been characterized as to its localization in the Cohn ethanol fractionation system(5), its further identification has been handicapped by the need for a sheep cell system to detect its presence. The following study was undertaken to eliminate the red cell from this system and to precipitate specifically the R.F., thus making it amenable to chemical and immunochemical study.

Methods. Lyophilized pooled human gamma globulin obtained from the American Red Cross dissolved in pH 8 buffered saline to 2.5 g % concentration was absorbed free of naturally occurring antibodies to sheep red cells and heated to 56°C for 30 minutes to inactivate complement. This material was used for coating the sheep red cells for the conventional Heller test and for the precipitation studies. It has been demonstrated previously that addition of extremely small quantities of this gamma globulin to a positively reacting serum from a patient with rheumatoid arthritis results in a negative FII test presumably by the interaction of the gamma globulin added with the R.F. of the patient's serum(2). The sera of subjects studied were complement inactivated and absorbed free of naturally occurring sheep cell antibodies. This treatment of the sera allowed both the precipitin test and the FII test to be done on the same specimen.[§] Nitrogen content of gamma globulin solutions was determined by the Kjeldahl method. Euglobulin was prepared by adding equal volumes of serum and distilled water in cellophane bag and dialyzing against distilled water for 48 hours at 4°C. The insoluble fraction (euglobulin) was washed with distilled water until washings were protein-free (Biuret), and redissolved in saline to serum concentration. Initial studies showed that when FII positive serum or its euglobulin fraction was

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[§] Precipitation occurs, however, even if the complement of serum and gamma globulin solution is not inactivated.

mixed with varying amounts of gamma globulin, a precipitate resulted. This precipitate formed immediately at the interface of the two components with the larger amounts of gamma globulin when an overlay technic was used. In all studies of precipitate formation, the tubes were first observed for precipitate at the interface, then mixed, allowed to stand at room temperature for 1 hour, placed at 4°C, and finally read by observing the precipitate while shaking the tubes. Aliquots of the supernatants were tested for excess of gamma globulin by the addition of the same serum, for excess rheumatoid factor by the FII test and by the addition of gamma globulin.

Results. Table I shows the result of combining a constant amount of an FII positive serum (0.5 cc of 1:7 dilution in pH 8.0 buffered saline) with varying amounts of gamma globulin. The plus sign indicates the presence of an opalescent gelatinous precipitate. Although quantitative immunochemical studies are not completed, this table suggests an equivalence zone with gamma globulin excess in the supernatant on one side and rheumatoid factor on the other(6).

Table II shows the result of simultaneous dilution of a serum euglobulin and of gamma globulin. Interpreted in the manner of a single antigen-antibody precipitin system, the rapid diminution in the amount of precipitate formed as the euglobulin was diluted (no precipitate at dilution 1:32), while precipitation still occurred with 200-fold dilution of gamma globulin, suggests that the latter or some component of it behaves as the antigen in this system(6).

TABLE I. Precipitation Reaction of Gamma Globulin with Rheumatoid Serum. Serum #145. 0.5 cc of 1:7 buffered saline dilution. FII titer > 56,000. 4°C for 4 days.

mg γ glob. N. in 0.5 cc	Degree of ppt. after 4 days at 4°C	FII test on supernatant	Excess γ globulin in supernatant	Excess R factor in supernatant
.440	4+	0	+	0
.408	"	0	+	0
.377	"	0	+	0
.346	"	0	+	0
.314	"	0	+	0
.283	"	0	+	0
.251	"	0	+	0
.220	"	0	+	0
.188	"	0	+	0
.157	"	0	+	0
.126	"	0	faint pos.	0
.0943	"	0	*Idem*	faint pos.
.0628	"	0	0	*Idem*
.0314	3+	896	0	"

TABLE II. Simultaneous Dilution of Gamma Globulin and Rheumatoid Serum Euglobulin. Dilution of serum euglobulin from a patient with rheumatoid arthritis (FII titer > 56,000) in buffered saline pH 8.0. 4°C for 48 hr. Precipitate measured on a scale of 0 to 4 plus.

mg γ glob. N. in 0.3 cc	Undil.	1:2	1:4	1:8	1:16	1:32	Buffered saline
.381	4+	3+	3+	2+	1+	0	0
.235	"	"	2+	"	"	±	0
.192	"	"	3+	"	2+	1+	0
.096	"	"	2+	"	1+	"	0
.048	3+	2+	"	1+	"	"	0
.0192	2+	"	"	"	"	±	0
.0096	1+	1+	1+	"	"	"	0
.0048	"	"	"	"	"	0	0
.00192	"	"	0	0	0	0	0
.00096	"	0	0	0	0	0	0
Buffered saline	±	0	0	0	0	0	0

The sera of 18 of 21 individuals with the clinical diagnosis of rheumatoid arthritis and a positive FII test showed precipitation under these conditions; no precipitation occurred with the other three. Of 27 individuals with a variety of joint diseases but with negative FII tests, none showed precipitation.

Summary. The precipitin reaction which has been observed will permit study of the 2 factors, one found in serum of patients with rheumatoid arthritis, the other in human gamma globulin, which interact to give a positive FII test (Heller). This reaction eliminates the need for sheep cells and lends itself to investigation by technics of quantitative immunochemistry(6).

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Growth of Human Epidermoid Carcinomas (Strains KB and HeLa) in Hamsters from Tissue Culture Inocula.[*] (22224)

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Eagle has reported the isolation of a human epidermoid carcinoma (strain KB) directly from a biopsy specimen(1) in a new medium which satisfies the specific amino acid and vitamin requirements of a mouse fibroblast (strain L) and the HeLa strain of epidermoid carcinoma(2-5). Since strain KB was isolated directly in these culture media without the intervening use of experimental animals, the purpose of the present report is to record its successful transplant from *in vitro* cultures to the cheek pouches of Syrian hamsters. Similar experiments with the HeLa cell also were undertaken, since this tissue culture strain apparently has not yet been studied in the hamster cheek pouch.

Methods. Strain KB, isolated Dec. 21, 1954 and maintained by serial subculture in these new media(1), and strain HeLa(6), similarly maintained in these media for more than a year, were obtained through the courtesy of Dr. Harry Eagle, National Institutes of Health. Both strains had been cultivated in these laboratories for some 4-6 months prior to the present experiments by regular subculture in the medium described by Eagle (2-5), which contains 13 essential amino acids, 7 essential vitamins, glucose and salts, each in the concentration necessary for maximal growth of these 2 cell lines(1-5). The essential serum protein was provided in the present experiments by 10% whole, fresh human serum. The layer of cells growing on the surface of the culture flasks was removed for subculture and for animal experiments by replacing the culture fluid in 8-10-day-old cultures with fresh media containing Difco "1:250" trypsin in a final concentration of 0.125%, and incubating the flask *circa* 5 min at 37°C. The dispersed cells were then sedimented at 5-600 rpm, washed 2 or 3 times with 10-15 ml of culture media to remove the trypsin and resuspended in a convenient volume of complete culture media. The suspensions were shaken gently to disperse the cells, and after enumeration by direct haemocytometer counts, diluted so that the desired number of cells was contained in a volume of 0.1 ml of media. Syrian hamsters, 60-80 g in weight, were prepared for implantation as described by Lutz *et al.*(7). The 0.1 ml of culture media containing the desired inoculum was implanted under the epithelium of the cheek pouch with a 24 gauge needle. Cortisone acetate in subcutaneous doses of 2-3 mg was administered simultaneously with implantation and twice weekly post-implantation(8).

Results. Strain KB. The implantation of 16,000 cells produced a visible nodule in the cheek pouch (Fig. 1a) following a latent period of 56 days. The nodules continued to grow and eventually filled the pouch, presenting the gross appearance of a typical cheek pouch tumor (Fig. 1b). The histology of these tumors[†] is consistent with that of an

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[†] We wish to thank Dr. J. Bernstein, Department of Pathology, Children's Medical Center, for histological study of these tumors.

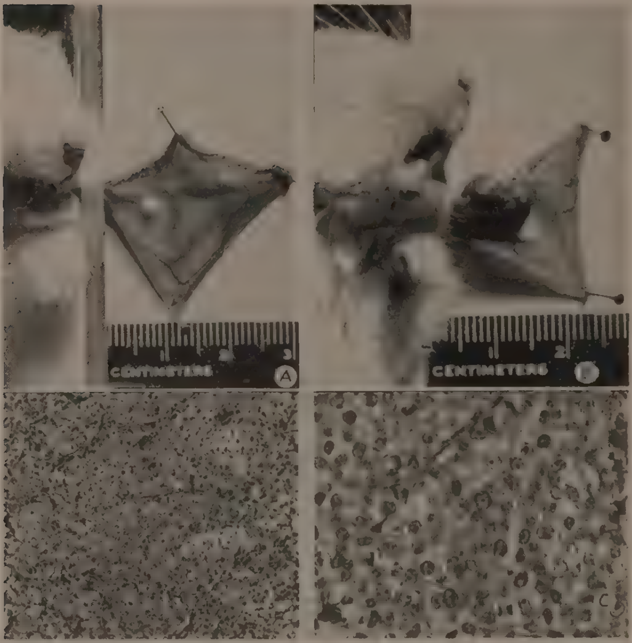

FIG. 1. Implantation of human epidermoid carcinoma, strain KB in hamster cheek pouch from tissue culture inocula. a. Implantation of 16,000 cells, 56 days. b. Same tumor, 76 days. c. Histologic section, 76 days. H. & E., 140 × and 440 ×.

epidermoid carcinoma (Fig. 1c). The latent period following implantation from *in vitro* culture has decreased progressively with successive hamster passages until now after the 12th passage, it must be transplanted every 10 days. The tumor grows in about 80% of the animals implanted, and if not transplanted on schedule, ulceration develops and the tumor becomes necrotic between 12-15 days. The animals then expire, usually as the result of secondary infection. Preliminary dose-response experiments done in duplicate with bilateral implantation in each animal indicate that the implantation of *circa* 15-20 cells results in the development of a tumor following a latent period of 153 days. Samples of tumor from the 9th passage of strain KB in cortisonized hamsters were implanted successfully in the cheek pouches of untreated animals. This tumor line now has been transplanted through 3 successive passages in non-conditioned hamsters. The tumor growth rate thus far is slower than in cortisone-treated animals. These tumors if not transplanted, undergo ulceration and necrosis between 22 and 25 days.

Tissue culture inocula of strain KB also have been implanted subcutaneously into LAF$_1$ mice bearing intramuscular transplants of the homologous ACTH secreting pituitary tumor reported by Furth *et al.*(9) according to the method described by Handler(10). Tumors developed in 80% of the mice bearing the pituitary tumor, attaining volumes of approximately 1 ml in thirty days. All mice died of the effects of the pituitary tumor. Thus far, strain KB has failed to grow in normal mice.

Strain HeLa. The implantation of 18,600 cells produced a nodule in the cheek pouch (Fig. 2a) following a latent period of 14 days. These nodules developed the gross appearance of typical cheek pouch tumors (Fig. 2b), the histology† of which is consistent with that of an epidermoid carcinoma (Fig. 2c). The latent period following implantation from *in vitro* culture has decreased to 10 days after 4 successive hamster passages. The tumor

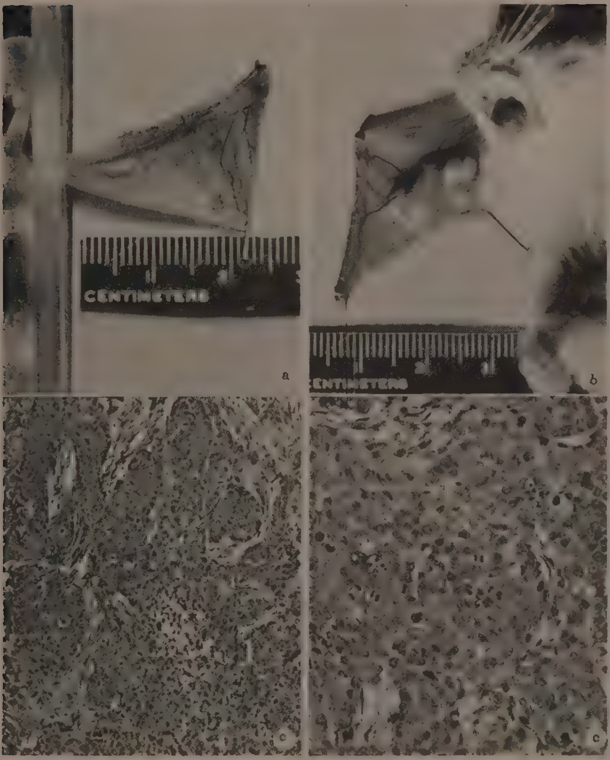

FIG. 2. Implantation of human epidermoid carcinoma, strain HeLa in hamster cheek pouch from tissue culture inocula. a. Implantation of 18,600 cells, 14 days. b. Same tumor, 34 days. c. Histologic section, 34 days. H. & E., 75 × and 220 ×.

grows in 60% of the animals implanted, and unless transplanted every 18 days, the cheek pouch ulcerates, the tumor becomes necrotic between 20-24 days and the animals die of secondary infection. Preliminary dose-response experiments done in duplicate with bilateral implantation in each animal indicate that the implantation of *circa* 15-20 cells results in the development of a tumor following a latent period of 92 days.

Discussion. The present experiments with strain KB represent the first instance in which a human tumor isolated directly from a biopsy specimen in these new media has been transplanted from cultures to experimental animals. Similarly, so far as is known, strain HeLa has not heretofore been implanted successfully in the hamster cheek pouch. These observations indicate a simple method by which various tumor cell lines isolated and/or maintained in these media can be checked periodically for tumor production *in vivo,* and further suggest the possibility that the same tumor cell lines may be established and used in parallel in these media and in the hamster cheek pouch for the comparative screening of potential chemotherapeutic agents.

The uniform tumors produced by measured tissue culture inocula should be particularly useful in the chemotherapeutic study of cheek pouch tumors. Quantitated cell suspensions prepared by the trypsinization of biopsy specimens(11) of certain other human tumors being carried in hamsters(12) have been equally effective when implanted into the hamster cheek pouch, as have been similar hamster and mouse tumor inocula when implanted appropriately in homologous hosts (12). These preliminary experiments also suggest that the latent period may be predetermined within reasonable limits by the number of tumor cells contained in the inoculum.

Summary. 1. The KB and HeLa strains of human epidermoid carcinoma have been established in the cheek pouches of golden hamsters by means of quantitated suspensions prepared from tissue cultures in a medium providing the specific amino acid and vitamin requirements of these cell lines. 2. Strain KB, isolated directly from a biopsy specimen in these media, has been grown in LAF_1 mice bearing homologous ACTH secreting pituitary tumors. This strain also has been established in the cheek pouch of non-conditioned hamsters but thus far has failed to grow in non-conditioned mice. 3. The use of quantitated tissue culture inocula for the production of tumors in the hamster cheek pouch for chemotherapeutic studies is suggested.

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Effects of Temperature and Work on Metabolism and Heat Loss in Man.*† (22225)

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Muscular exercise in a cold environment by either man(1) or animals(2,3) requires a greater oxygen consumption than the same activity in a warm environment. In man, the added energy requirement for work in the cold is not entirely the added hampering effect of clothing(4). Hart(2) concluded that the extent to which heat produced by activity is used to maintain body temperature depends upon the effect of activity on over-all physiological body insulation. The following experiments were devised to disclose changes of insulation in man exercising in warm and cool environments.

Methods. Skin temperature, heat loss and oxygen consumption were measured in 4 male subjects clad in shorts and exercising in cooling and cool environments. The experiments were begun at an ambient temperature of 20°C. The temperature was then steadily decreased to 10°C over ½ hour, and maintained at 10°C during the remainder of the test period (30 to 60 minutes). The subject reported shivering on a 4-point scale. Exercise on a bicycle ergometer was performed at a rate of 150 kg m/min or 70 kg m/min. Exercise was initiated with the start of cooling in some experiments, and after shivering had begun as a result of cooling in others. Skin temperature was measured with copper-constantan thermocouples recorded by a Brown

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potentiometer with a –0.5 to 2.5 mV range. Thermocouples were placed according to the recommendations of Hardy and Du Bois(5), and the thermocouples were paralleled to give an average reading. Heat loss was recorded with Hatfield-Turner discs(6), 4 in series on the thorax and 4 in series on the thigh and leg. Oxygen consumption was measured with a laminar flowmeter(7) with a proportionate sample analyzed in a Pauling oxygen meter, range 110 to 160 mm Hg.

Results. The time course of the parameters measured is given in Fig. 1 and 2, and summarized in Fig. 3. When exercise was initiated during cooling (Exercise During Cooling, Item I, Table I), the skin temperature dropped at a faster rate over the initial 30 minute period than it did during cooling at rest (Item 2, Table I), although the final average skin temperature was slightly higher. The heat debt, calculated on the basis that ⅓ of the body weight was shell, was less when the subject exercised during cooling. Exercise during cooling was accomplished with an oxygen consumption comparable to that required when exercise was performed at 20°C (Item 4, Table I). Rectal temperature re-

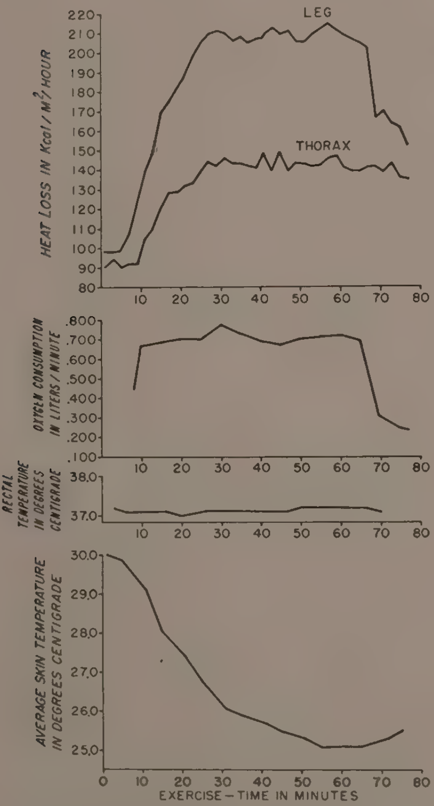

FIG. 1. Change in rectal temp., avg skin temp., oxygen consumption and heat loss with time. Cooling started at 5 min. Temperature changed from 20°C to 10°C over a ½ hr period and maintained at 10°C the remainder of the test period. Exercise started at 5 min. In the discussion this experiment is referred to as exercise during cooling. Avg of 4 subjects.

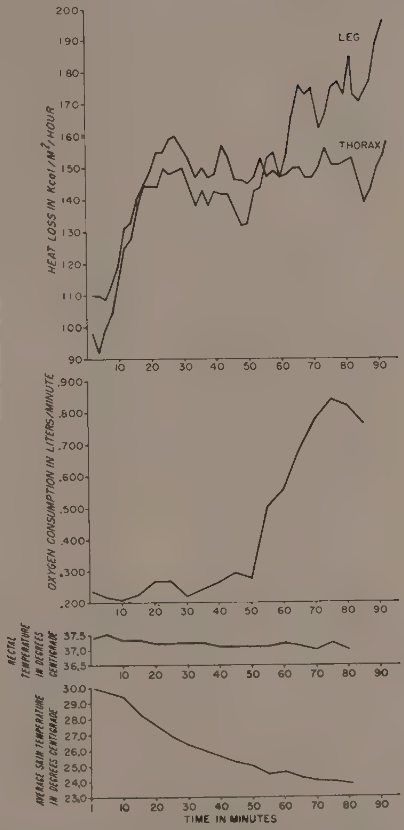

FIG. 2. Change in rectal temp., avg skin temp., oxygen consumption and heat loss with time. Cooling started at 5 min. Temperature changed from 20°C to 10°C over a ½ hr period and was maintained at 10°C for remainder of test period. Exercise was initiated at 50 min. In the discussion this experiment is referred to as rest during cooling and exercise after cooling. Avg of 4 subjects.

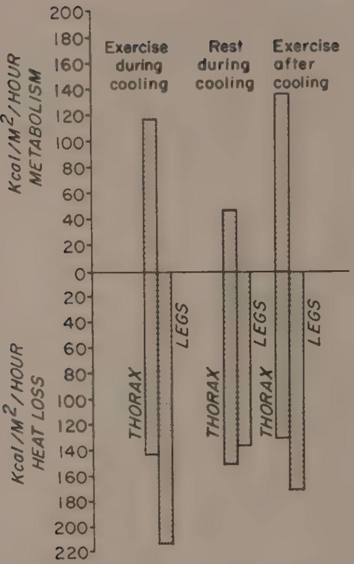

FIG. 3. Avg values for heat production plotted above horizontal line, and heat loss from the thorax and legs plotted below horizontal line. Heat loss from legs with exercise during cooling is greater than with exercise after cooling, and energy requirement when exercise is initiated after cooling is greater than when exercise is accomplished during cooling.

mained unchanged during the experiment when there was exercise during cooling, but fell slightly during cooling at rest.

Heat input increased when exercise was initiated after cooling, and the heat loss from the legs was not as great in this circumstance as when there was exercise during cooling. The increase in heat loss from the legs with exercise reflects the change in convective losses as well as the increased leg skin temperature. If the air insulation, Ia, is calculated for thorax and thigh plus leg according to the equation $Ia = \frac{Ts - Ta}{H}$ (8) this value for the thorax in the 2 experimental conditions is 0.12°C/cal/hr/m^2. The value of Ia for the legs is 0.105 during shivering and 0.067 to 0.081 during exercise.

Fall in rectal and skin temperature was arrested during exercise, but skin temperature did not rise during 20 min. of exercise. Increased heat loss via respiration is estimated to be of the order of 20% greater when exercise was initiated after shivering (29 kg cal/hr vs. 24 kg cal/hr).

Even after 90 min of shivering (Item 3, Table I), a steady state was not reached, and the heat input was approximately doubled. The skin and rectal temperatures were still slowly declining. The heat output did not balance with the heat input. At this time, the metabolism was 48 kg cal/hr/m^2 above the resting level at 20°C. Exercise, which at 20°C required a heat input of 45 kg cal/hr/m^2, required an additional 76 kg cal/hr/m^2 and then did not stop shivering in 30 min.

The drop in skin temperature may be used to calculate the heat debt on the basis that ⅓ of the body weight was cooling at this rate with a specific heat of 0.83. If the skin temperature decline is divided into 2 phases, the initial rapid decline and the subsequent slower fall, the heat debts may be compared. During cooling at rest heat debt was initially incurred at the rate of 75 kg cal/hr/m^2, and during cooling with exercise, at 93 kg cal/hr/m^2. In the second phase, skin temperature dropped an average of 3.9°C an hour at rest, and only 2.1°C an hour during exercise. Calculated on the same basis, this represents a rate of heat loss of 34 kg cal/hr/m^2 at rest and 18 kg cal/hr/m^2 during exercise. The area represented by the Hatfield-Turner discs on the thorax was assumed to be 0.35 body surface, and the area represented by the discs on the leg was assumed to be 0.32 body surface; Table II was then constructed. Considering the perils of the assumptions, the agreement of values obtained during exercise is particularly good.

Discussion. It appears from these data that the energy cost of exercise is greater after than during cooling. The reason is not demonstrated. Shivering usually continues during the exercise. The over-all direct heat loss, as indicated by the Hatfield-Turner discs, was less when exercise was performed after cooling. It would appear that the control of peripheral circulation exerted by exercise is modified by prior cooling. There may be an increased work related to the changes in temperature and motility that occur in muscles

TABLE I. Metabolic Changes with Exercise at Low Temperature.

	Rate of cooling, °C/min.	Time to shiver, min.	Skin temp., °C Initial	Skin temp., °C Final	Heat debt*	Heat input,† rest, 20°C	Heat input, shivering	Heat input, exercise	Heat loss, shivering	Heat loss, exercise	Heat loss, shivering	Heat loss, exercise
					kg cal/hr/m²							
Item 1												
Exercise during cooling	.16	—	30.2	25.5	49			122		145		223
	.13	—	29.8	24.5	57			112		149		183
	.14	—	29.8	24.7	53			110		146		224
	.17	—	30.5	25.5	64			120		132		206
Avg	.15	—	30.1	25.0	56	36		117		143		214
Item 2												
Cooling at rest; exercise after	.16	13	30.8	24.7	63		48	140	151	147	136	174
	.11	49	30.0	23.9	74		37	133	126	130	122	147
	.12	34	29.9	24.2	66		60	132	143	149	142	185
	.10	66	29.7	23.3	83	36	37	135	~~181~~	160	149	179
Avg	.12	48	30.1	24.3	72		46	135	151	147	137	171
Item 3												
Same as 1 & 2 (see ‡)	.18	33–48	31.5	25.0	67			83‡		182		223
	.15	25	30.7	25.0	60	35	79	111‡	170	168	134	189
Item 4												
Exercise at 20°C	0							107				
	0							80‡				

* Calculated on basis of 1/3 body cooling at skin temperature and 2/3 at rect. temperature.

† " " " " caloric value of liter of $O_2 = 4.825$.

‡ Exercise at 70 kg M/min., others at 150 kg M/min.

TABLE II. Heat Balance Calculations.

Item	Heat loss, kg cal/hr		Heat input, kg cal/hr					
Item 1	Thorax	94	Metabolism	221				
	Legs	128	Skin temp.	42				
	Remainder	41	Rectal	0				
		263		263				
Item 2	Heat loss (shivering), kg cal/hr		Heat input (shivering), kg cal/hr		Heat loss (exercise), kg cal/hr		Heat input (exercise), kg cal/hr	
	Thorax	100	Metabolism	87	Thorax	97	Metabolism	255
	Legs	82	Skin temp.	79	Legs	105	Skin temp.	0
	Remainder	0	Rectal	16	Remainder	49	Rectal	–4
		182		182		251		251

and joints(9,10). In these experiments, the steady state was not reached during the first 2 or 3 hours of cooling. Metabolism was eventually doubled at 10°C. In these experiments skin temperature dropped at a rate comparable to that reported by Horvath *et al.* (11), whose subjects were well-clothed individuals exposed to –40°C with a heat input of 74 kg cal/hr/m² at 2 hours' and 85 kg cal/hr/m² at 3 hours' exposure. The increase in heat input with exercise is of the same order of magnitude as that reported by Horvath for clothed men.

Summary. Exercise after cooling is accomplished at a greater energy cost than exercise during cooling. Over-all direct heat loss is less during exercise after cooling.

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Phosphatase Activity of Hematopoietic Tissues of Nitrogen Mustard-Treated Animals.* (22226)

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Previous studies in this laboratory(1) have shown that whole body x-irradiation markedly inhibits citric acid synthesis *in vivo* by the spleens and thymus glands of rats and mice. Treatment of animals with nitrogen mustards caused a similar inhibitory effect on citric acid formation by hematopoietic tissues (2). More recently we found(3) that sublethal and lethal doses of x-irradiation produce marked increases in the adenosine triphosphatase and 5-nucleotidase activity of the spleens and thymus glands. In view of the known similarities between x-irradiation and the nitrogen mustards in some of their actions on mammalian tissues we were interested in ascertaining whether these chemical agents produced changes in phosphatase activity similar to those which

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result from x-irradiation. To test this possibility various doses of the hydrochlorides of methyl *bis*(2-chlorethyl)amine (HN2) and ethyl *bis*(2-chlorethyl)amine (HN1) were administered intraperitoneally to rats and the adenosine triphosphatase and 5-nucleotidase activity of the spleens and thymus glands was measured at various intervals. Some measurements of the adenosine triphosphatase activity of the tissues of mice treated with HN2 were also made. The results of these experiments demonstrated that the nitrogen mustards resemble x-irradiation(3) in their effects on the phosphatase activity of hematopoietic tissues.

Materials and methods. Young male Sprague-Dawley rats (175-225 g) and male Carworth Farms mice (20-30 g) were used for this study. The animals were maintained in an air-conditioned laboratory at 65-75°F and were fed Rockland Rat Diet. Freshly prepared aqueous solutions of HN1 and HN2 were administerd intraperitoneally. For the enzyme assays the animals were sacrificed by decapitation at intervals after treatment with the nitrogen mustards and cold, aqueous homogenates of spleen and thymus glands were prepared. The adenosine triphosphatase assays were performed by the method of DuBois and Potter(4) using 1% homogenates of mouse spleen, mouse thymus glands and rat thymus glands and 0.5% homogenates of rat spleen. Duplicate assays using 2 levels of tissue were made. For the measurements on rat spleen 0.5 mg and 1 mg of tissue were used and for the assays on the thymus glands of both species and mouse spleen 1 mg and 2 mg of tissue were employed. The adenosine triphosphatase activity was expressed as units, *i.e.*, the micrograms of inorganic phosphorus liberated from adenosine triphosphate/mg of tissue during a 15-minute incubation period. The 5-nucleotidase assays were performed using a method developed in this laboratory(5). For measurement of the 5-nucleotidase activity of rat spleen and thymus glands 2% homogenates were prepared and quantities of homogenate containing 2 mg and 4 mg of tissue were used. Inorganic phosphorus measurements were made by the method of Fiske and SubbaRow(6).

Results. In a previous study in which HN1 and HN2 were administered to rats under conditions comparable with those used for the present experiments the LD_{50} of the hydrochloride of HN1 was 0.75 mg/kg and LD_{50} for HN2 hydrochloride was 1.5 mg/kg when the compounds were given intraperitoneally (2). To measure the influence of HN2 on the adenosine triphosphatase activity of the spleens and thymus glands of rats sublethal doses of 0.5 mg/kg and 0.75 mg/kg were given to 2 series of animals. At daily intervals for 5 days 4 animals at each dosage level were sacrificed for adenosine triphosphatase measurements on the spleens and thymus glands. The results of these measurements are shown in Fig. 1 in which each value on the curves is the average for assays on the tissues of 4 animals.

Administration of 0.75 mg/kg of HN2 hydrochloride resulted in an increase in the adenosine triphosphatase activity of the spleen to 186% of normal in 2 days. The spleens of rats which received 0.5 mg/kg of HN2 hydrochloride exhibited a maximum increase to 161% of normal in 3 days. Gradual reversal of the alteration in enzyme activity followed the initial increase and the rate of reversal was more rapid with the lower dose of HN2. The thymus glands exhibited a much smaller increase in enzyme activity during the observation period after these doses of the nitrogen mustard. However, in other experiments in which higher doses of HN2 were administered considerable increases in the adenosine triphosphatase activity of the thymus glands were noted. Thus, after 2 mg/kg of HN2 hydrochloride the adenosine triphosphatase activity of the thymus glands increased to 179% of normal in 3 days.

TABLE I. Comparison of the Increase in Adenosine Triphosphatase Activity of Spleens of Rats 72 Hours after Various Doses of HN1 and HN2.

Nitrogen mustard (mg/kg)	Adenosine triphosphatase activity (μg P/mg tissue/15 min.) HN1	HN2
.5	36.4 (34.3-38.0)	27.5 (26.4-28.9)
.75	44.6 (40.1-46.5)	30.3 (28.2-32.5)
1.0	47.4 (43.5-49.6)	35.6 (34.4-37.6)

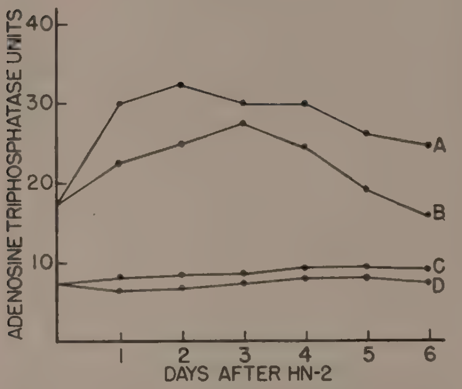

FIG. 1. Effect of methyl *bis*(2-chlorethyl)amine (HN2) hydrochloride on adenosine triphosphatase activity of spleens and thymus glands of rats. A, spleen 0.75 mg/kg; B, spleen 0.5 mg/kg; C, thymus glands 0.75 mg/kg; D, thymus glands 0.5 mg/kg.

To ascertain whether a relationship exists between the toxicity of nitrogen mustards and their ability to increase the adenosine triphosphatase activity of the spleens of rats a comparison of the effects of equivalent doses of HN1 and HN2 was made. For these measurements rats were given 0.5, 0.75 and 1 mg/kg of the hydrochlorides of HN1 and HN2 intraperitoneally. Groups of 4 animals at each dosage level were sacrificed at 72 hours for adenosine triphosphatase measurements on the spleens. The results of these measurements are shown in Table I in which the average and the range of values for each group are presented. The amount of increase in adenosine triphosphatase activity produced by each of the nitrogen mustards was dependent upon the dose and HN1 was more effective than HN2 at comparable dosage levels in agreement with the higher toxicity of HN1 to rats.

The effects of HN2 on the adenosine triphosphatase activity of mouse tissues was measured after administration of single doses of 2, 5 and 10 mg/kg of the hydrochloride intraperitoneally. The results of adenosine triphosphatase measurements conducted at daily intervals on the spleens and thymus glands are summarized in Fig. 2 in which each value on the curves is an average for at least 4 animals. At 3 days after administration of 5 and 10 mg/kg of HN2 hydrochloride the adenosine triphosphatase activity of the spleens of mice increased to 165% and 186% of normal respectively. Similar increases were noted in the thymus glands. The respective mean values and ranges for the spleens of mice receiving the 2 doses of HN2 were 18.2 (15.7-19.9) and 20.5 (17.4-23.6) adenosine triphosphatase units. Statistical analysis of the difference between these values yielded a *t* value of 2 indicating that the difference is of doubtful significance. Thus a dose of 5 mg/kg produced the maximum increase in the adenosine triphosphatase activity which can be induced by HN2 in the spleens and thymus glands of mice. A similar effect was observed in rats in that doses of 2.5 and 5 mg/kg of HN2 hydrochloride caused essentially the same amount of increase in the adenosine triphosphatase activity of the hematopoietic tissues. Dose-dependent increases in phosphatase activity are, therefore, observed mainly in the sublethal dosage range after administration of the nitrogen mustards or exposure to x-ray(3). Another series of mice received 2 mg/kg of HN2 hydrochloride which is sublethal for this species. The adenosine triphosphatase activity of the spleen increased to an average value of 156%

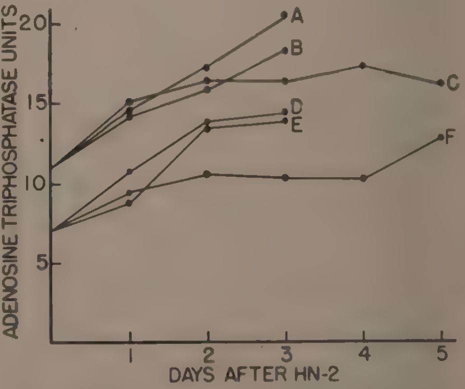

FIG. 2. Influence of methyl *bis*(2-chlorethyl)amine (HN2) hydrochloride on adenosine triphosphatase activity of spleens and thymus glands of mice. A, spleen 10 mg/kg; B, spleen 5 mg/kg; C, spleen 2 mg/kg; D, thymus glands 10 mg/kg; E, thymus glands 5 mg/kg; F, thymus glands 2 mg/kg.

of normal in 4 days after which time a gradual reversal toward normal levels occurred.

Measurements of the influence of HN2 on the 5-nucleotidase activity of the spleens and thymus glands of rats were also conducted. For this experiment a series of rats was given 0.5 mg/kg of HN2 hydrochloride intraperitoneally and groups each containing 4 animals were sacrificed at daily intervals for 5 days. The 5-nucleotidase activity of the spleens increased to 153% of normal in 4 days and an increase to 167% of normal was noted in the thymus glands at 3 days after administration of the nitrogen mustard. Gradual return of the enzyme activity to normal levels followed the initial increase in activity. The intraperitoneal administration of 0.75 mg/kg of HN2 hydrochloride caused an increase in the 5-nucleotidase activity of the spleen to 173% of normal and an increase in the enzyme activity of the thymus glands to 149% of normal.

Discussion. Results of this investigation demonstrated that sublethal doses of the nitrogen mustards produce increases in the adenosine triphosphatase and 5-nucleotidase activity of the spleens and thymus glands. The amount and duration of the increase in enzyme activity was dependent upon the dose of the nitrogen mustards. Higher doses of HN2 were necessary to produce increases in the adenosine triphosphatase activity of the hematopoietic tissues of mice than rats in agreement with the higher toxicity of this nitrogen mustard to rats. At equivalent dosage levels HN1 produced a greater increase in adenosine triphosphatase activity than HN2 thus indicating a relationship between the toxicity of various nitrogen mustards and their actions on phosphatase activity *in vivo*. After sublethal doses of the nitrogen mustards the increase in enzyme activity was reversible. The effects of the nitrogen mustards on the phosphatase activity of the spleens and thymus glands resembled qualitatively the effects of x-ray on the phosphatases of these tissues. This relationship was evident with respect to time of onset, magnitude and duration of the increase in enzyme activity. However, from a quantitative standpoint x-ray was more effective in terms of the LD_{50} values than the nitrogen mustards in increasing the phosphatase activity of hematopoietic tissues.

Previous studies in this laboratory have demonstrated(1,2) a qualitative similarity between the ability of the nitrogen mustards and x-ray to inhibit citrate synthesis in hematopoietic tissues of rats. On the basis of the available evidence it is not possible to state whether the defect in citric acid synthesis is related to the alteration in phosphatase activity. Both of these biochemical changes occur at essentially the same time and persist for approximately equal periods after treatment of animals with x-ray or a nitrogen mustard. The exact mechanism involved in the production of increased phosphatase activity in the hematopoietic tissues produced by these toxicants is unknown. On the basis of histochemical findings Ackerman *et al.*(7) suggested that the radiation-induced increase in phosphatase activity of the spleen is due to a relative increase in the red pulp and clasmatocytes as well as an actual increase in the phosphatase of the clasmatocytes. The similarity between the nitrogen mustards and x-ray which was demonstrated in the present investigation indicates that either of these agents may be employed in future studies on the exact mechanism responsible for the increased phosphatase activity of hematopoietic tissues.

Summary. The effects of nitrogen mustards *in vivo* on the phosphatase activity of the spleens and thymus glands of rats and mice were studied. The results of this study indicated that sublethal doses of HN2 produce dose-dependent increases in the adenosine triphosphatase activity and the 5-nucleotidase activity of the spleens and thymus glands with the maximum increase being observed at 3 or 4 days after intraperitoneal administration of the compound. Reversal of the increase in enzyme activity was noted after sublethal doses of HN2. At equivalent dosage levels HN1 was more toxic and more effective than HN2 in increasing the adenosine triphosphatase activity of the spleens and thymus glands of rats.

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Biotin Derivatives in Human Urine. (22227)

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The nature of the biotin derivatives that occur in urine has been the subject of a number of studies since Snell *et al.* (1) first showed that urine is a relatively rich source of the vitamin. Oppel(2) concluded that there exist in urine two forms of biotin, one avidin combinable and one avidin non-combinable. Burk and Winzler(3) postulated the existence in natural material (including urine) of a number of biotin derivatives (miotin, tiotin, and rhiotin) distinguishable by differences in microbiological activity, chemical stability, or avidin combinability. Chu and Williams(4), on the other hand, could not detect the presence in any natural material other than urine of avidin non-combinable forms of biotin. They even seriously questioned the real validity of their own data concerning the existence in urine of small amounts of such a derivative(s).

The above described studies were carried out primarily with *Saccharomyces cerevisiae* as the assay organism. Since yeast growth is notoriously influenced by a variety of stimulatory and inhibitory materials of unknown nature, the specificity of the responses described above with respect to any real relationship to biotin may be questioned. Significant to this discussion also is the fact that none of the compounds whose existence was postulated in the references cited has ever been obtained in the pure or even highly concentrated state. On the contrary, a number of other biotin derivatives have been derived from natural material or products derived from natural material and have been adequately characterized. These derivatives are biocytin (ϵ-N-biotinyl-L-lysine)(5), biotin d-sulfoxide(6-8), and biotin l-sulfoxide(9-11). A number of other biotin derivatives including biotinamide(12,13), oxybiotin(14), and N-biotinyl derivatives(12,13) of glycine, β-alanine, L-aspartic acid, L-glutamic acid, L-leucine, and p-aminobenzoic acid have been synthesized in the laboratory and studied microbiologically. Differential microbiological assays with a high order of specificity in some instances are now available for studies with these compounds. In combination with the bioautographic technic, such differential microbiological asays can yield considerable information concerning the nature of the biotin derivatives in a given sample. One study(15) employed butanol-acetic acid-water as the developing system and *Neurospora crassa* as the indicating organism. The butanol-acetic acid-water system is remarkably free of complications from salts and other components of natural material. The organism *Neurospora crassa* is extremely versatile as far as response to biotin derivatives is concerned. The following compounds are essentially equally active: Biotin, oxybiotin, biotinamide, desthiobiotin, biotin d-sulfoxide, biotin l-sulfoxide, biocytin, biotinyl β-alanine, biotinyl glycine, biotinyl aspartic acid, and biotinyl leucine(10,12,13,16). In addition, R_F data obtained with *Neurospora crassa* are available for the mixed sulfoxides or the individual d- and l-sulfoxides in some instances of biocytin, biotinyl β-alanine, biotinyl glycine, biotinyl aspartic acid and biotinyl leucine which are formed when these compounds are paper chromatographed in those solvents which lead to the similar formation of biotin d-sulfoxide and biotin l-sulfoxide from biotin(15,16). Pimelic acid and related dicarboxylic acids are inactive microbiologically. On the other hand, *Lactobacillus arabinosus* is highly specific with respect to its requirement for biotin. Oxybiotin, N-biotinyl glycine, and biotin d-sulfoxide are the only compounds among the biotin derivatives presently available with more than 5% of the activity of biotin in promoting growth of this organism. The difference btween the "biotin" content of a particular sample determined with *Neurospora crassa* and that obtained with *Lactobacillus arabinosus* would appear then to have some significance as a measure of derivatives of biotin. The cyclic urea ring of biotin is essential for avidin combinability. Biotin derivatives containing such a structure combine with avidin with widely varying but characteristic affinities that are always less than that found with biotin itself(17,18). Cognizance of the above considerations has been made in the present studies on the biotin content of human urine.

TABLE I. Biotin Derivatives in 4 Urine Samples.

''Total'' biotin by *Neurospora crassa* assay, γ/ml	''Free'' biotin by *Lactobacillus arabinosus* assay, γ/ml	Unknown biotin derivative by difference, γ/ml
.018	.016	.002
.018	.017	.001
.014	.012	.002
.029	.024	.005

Materials and methods. "Free" biotin was determined in the urine samples with *Lactobacillus arabinosus* (ATCC 8014) as described previously in detail(19,20). "Total" biotin was determined with *Neurospora crassa*(9,11). The bioautographic procedures also have been adequately described previously(9,11,15). In the present studies 0.2 ml of urine (0.02 ml applied 10 times) or 0.02 γ of biotin or biocytin as biotin (0.02 ml of a 1 γ/ml solution) or 2 mg of urea (0.02 ml of a 20 mg per ml solution applied 5 times) was chromatographed on Whatman No. 1 paper where the solvent phase was obtained from the equilibration of butanol(5), water(4), and acetic acid(1), and the aqueous phase was present in a separate receptacle. After removal of the solvent by air drying in the hood the paper chromatograms intended for bioautography were cut into 21 sections such that each section represented 0.05 of an R_F unit. These sections were individually eluted in Erlenmeyer flasks with 25 ml of basal *Neurospora crassa* medium. After removal of the paper sections following a 1-hour elution period the flasks were plugged, autoclaved, seeded with *Neurospora crassa,* and incubated at 30° for 3-4 days. The extent of growth of the mold was determined by collecting the mycelium on a wire loop, squeezing to incipient dryness, and then drying to constant weight at 80°C (usually 18 hours). When the mycelium weight was plotted as a function of the paper section number a smooth curve connecting the points permitted localization of the R_F values by interpolation to within 0.02 unit. Urea was located on paper chromatograms by spraying with 2% p-dimethylaminobenzaldehyde in 1 N HCl. The avidin combinability of the biotin derivatives in urine was determined with *Neurospora crassa.* A microbiological assay using the usual 8 flasks of biotin standard, 2 series of 4 flasks containing increasing levels of urine, and 2 series of 8 flasks each containing a constant amount of the 2 urine samples studied was prepared. After the usual addition of medium, autoclaving, and seeding, 8 increasing levels of a sterile egg white dilution as a source of avidin were added in duplicate to the series of flasks to which the constant levels of urine had been added previously. After growth of the organism the weight of mycelium in each flask was determined in the usual way.

Results. As indicated by the microbiological assay data of Table I human urine contains in addition to free biotin, or material with microbiological activity for *Lactobacillus arabinosus,* small amounts of a biotin derivative measurable with *Neurospora crassa.*

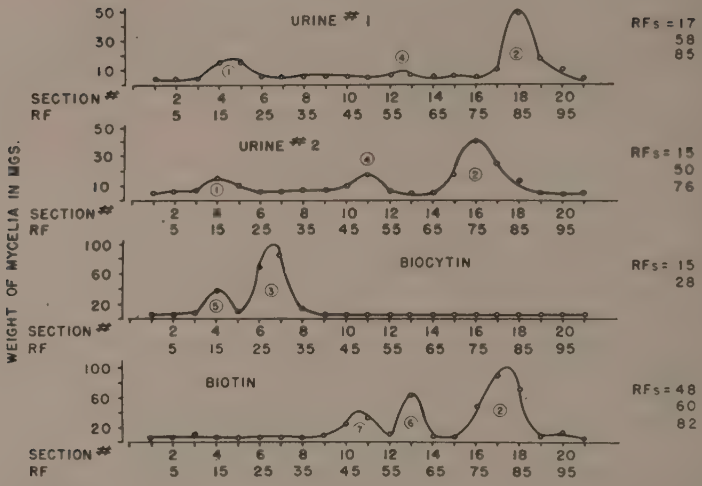

FIG. 1. Summary of bioautographic data. The various peaks are interpreted as follows: (1) unknown biotin derivative, (2) biotin, (3) biocytin, (4) biotin "sulfoxide," (5) biocytin "sulfoxide," (6) biotin d-sulfoxide, (7) biotin l-sulfoxide.

The paper chromatographic data of Fig. 1 confirm the predominant form of biotin in urine measurable with *Lactobacillus arabinosus* as free biotin (R_F ca 0.83). Indicated also by the chromatograms is the presence of biotin d-sulfoxide. This compound together with much smaller amounts of biotin l-sulfoxide (not present in detectable amounts in the present urine chromatograms) is encountered when biotin is paper chromatographed on Whatman No. 1 paper in the butanol-water-acetic acid system. Its presence in the chromatogram when biotin is also present does not necessarily mean that it was originally present in the urine samples studied. The paper chromatograms do indicate the existence in urine of small amounts of a biotin derivative with a low R_F value. The unknown material has an R_F value in the butanol-water-acetic acid system corresponding quite closely to that of the two sulfoxides of biocytin which are not resolved in this solvent or other solvent systems studied. The corresponding sulfoxide of biocytin to which the present biotin derivative conceivably may be related is unknown, and accordingly is referred to hereafter as "biocytin sulfoxide." R_F values for the available N-biotinyl derivatives of glycine, β-alanine, aspartic acid, and leucine as well as the corresponding sulfoxides are all considerably higher(16). Attempts to demonstrate correspondence of R_F values of the unknown component of urine and "biocytin sulfoxide" in a number of other solvent systems have not been conclusive since it became apparent that the R_F values observed in these additional solvents were influenced to some extent by the extraneous components of urine. A certain amount of concentration and purification probably is indicated before paper chromatographic data would be conclusive regarding the identity of the biotin derivative of urine with "biocytin sulfoxide." It seems unlikely that, if the unknown derivative indicated on the chromatograms is "biocytin sulfoxide," it arose from an *in vitro* oxidation of biocytin by the filter paper for the following reasons: (a) it is present in urine prior to any contact with filter paper as indicated by the differential microbiological assays, and (b) biocytin, from which "biocytin sulfoxide" might conceivably have arisen by a certain amount of *in vitro* oxidation analogous to that observed with biotin, was entirely absent as indicated by the chromatograms. Biocytin would not be expected to occur in urine be-

TABLE II. Avidin Combinability of Biotin Derivatives in Urine.

Biotin, γ	Urine, ml	Egg white (avidin), ml	Mycelium, mg
0			1.0
.0005			11.6
.0010			19.7
.0015			27.7
.0020			31.9
.0030			42.6
.0050			45.0
	.02 No. 1		13.2
	.04		22.2
	.06		30.0
	.10		42.1
	.02 No. 2		10.0
	.04		17.3
	.06		24.9
	.10		36.8
	.1 No. 1	.002	24.6
	.1	.004	4.0
	.1	.006	1.5
	.1	.01	0
	.1	.02	0
	.1	.04	0
	.1	.06	0
	.1	.10	0
	.1 No. 2	.002	15.4
	.1	.004	3.2
	.1	.006	.7
	.1	.01	0
	.1	.02	0
	.1	.04	0
	.1	.06	0
	.1	.10	0

cause of the rapid hydrolysis of biocytin by an enzyme of blood (and presumably other tissues)(21). If the unknown component of urine is not "biocytin sulfoxide" it is at least a derivative considerably more "hydrophilic" than biocytin. Urea in lieu of biotin was found to be inactive for *Neurospora crassa* and to have an R_F value in the butanol-acetic acid-water system high enough (0.46) to eliminate it from consideration as a nonspecific stimulant. As indicated by the data of Table II all the biotin in urine determinable with *Neurospora crassa* is combinable with avidin. These results would indicate that the unknown derivative contains a cyclic urea ring.

The best interpretation of the data described above is that with a biotin auxotroph considerably more versatile with respect to utilization of biotin derivatives than microorganisms previously employed but at the same time considerably more specific with respect to the response being due to material with a structural or functional relationship to biotin, no evidence is available for the existence in human urine of any "unusual" biotin derivatives. Although an unknown form of biotin is present the compound on the basis of paper chromatographic data could very well be "biocytin sulfoxide." No evidence is available for the existence of "avidin uncombinable" forms of biotin.

The isolation and characterization of the derivative according to classical procedures would be a formidable undertaking. Since the factor is present in urine only to the extent of about 2 γ per liter (provided, of course, that its microbiological activity is equal to that of biotin), to obtain the 100 mg that might be required for characterization studies, it would be necessary to work up at least 50,000 liters of urine. These calculations assume 100% recovery of the factor. If, as is more usual in such isolations, the recovery were to run 1 to 10%, the processing of between 500,000 and 5,000,000 liters of urine would be required for successful completion of the problem. When calculated in another way the factor may be described as present to the extent of only about 0.04 γ per g of urinary dry matter. If urine is the only source of the factor a comparison of the occurrence of this factor with the occurrence of other "trace factors" reveals that this compound is probably one of the most sparsely distributed of any biochemical compound presently known.

Summary. Microbiological experiments involving direct assays as well as bioautographic procedures indicate that human urine contains relatively large amounts of free biotin and very small amounts of a "hydrophilic" biotin derivative that may be "biocytin sulfoxide." No evidence was found for the existence of other "unusual" biotin derivatives. All the biotion of urine determinable by an organism more versatile in its response to biotin derivatives than any studied previously is avidin combinable.

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Interaction of Gelatin-Stabilized Radiogold Colloid and Plasma Proteins. (22228)

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It has been demonstrated that the rate of disappearance of gelatin-stabilized radiogold colloid (Aurcoloid) from the circulation is inversely proportional to the amount of gelatin used to stabilize the Aurcoloid(1). Furthermore, the gelatin effect was enhanced by either injection of homologous rat plasma or alpha and beta globulin fractions of human plasma, whereas human serum albumin and gamma globulin had little effect. There was no evidence to suggest that the phagocytic capacity of the reticulo-endothelial system (RES) for the gold particles had been altered by gelatin. The present study was undertaken to investigate the interactions of gelatin with the plasma proteins as a possible explanation for this effect of gelatin.

Materials and methods. The Aurcoloid* was diluted with 50 volumes of distilled, demineralized water and stored in a refrigerator. The P-111 gelatin† solution, similar to that used by the manufacturer to stabilize the Aurcoloid, was handled under sterile conditions and maintained at 37°C in a water bath for at least 15 minutes before use. For the *in vitro* experiments, blood was obtained from adult male rats of the Wistar strain via the abdominal aorta using heparinized syringes. Human blood was obtained by venipuncture using heparinized syringes. After centrifugation, 3 ml volumes of plasma were placed in test tubes to which was added 0.05 ml of the diluted Aurocoloid and 2.5 mg of gelatin. Plasma samples were agitated for 2 minutes, incubated at 37°C for 30 minutes, then electrophoretically separated. In the *in vivo* experiments, adult male rats of Wistar strain weighing between 200-300 g were used. Animals were anaesthetized by intraperitoneal injection of nembutal, 5 mg/100 g of body weight. The external jugular veins were exposed and injections of the Aurcoloid

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* Obtained from Abbott Laboratories, Oak Ridge, Tenn.

† Obtained from Charles B. Knox Gelatin Products, Camden, N. J.

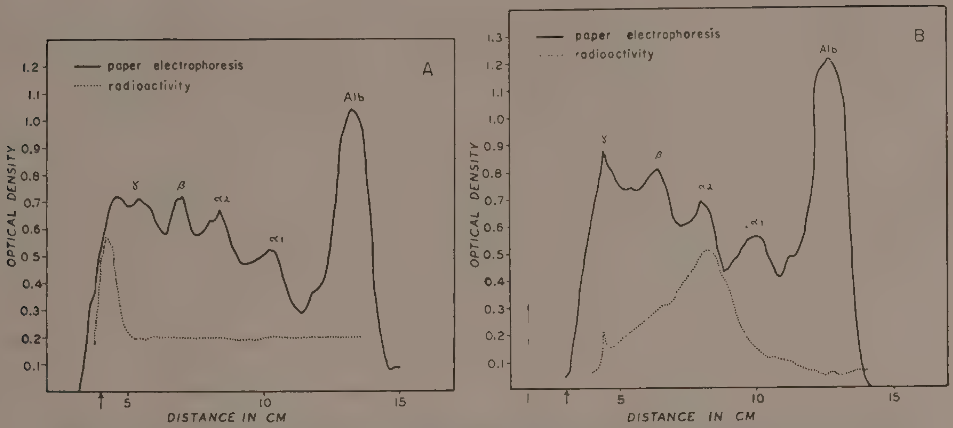

FIG. 1. Gelatin-plasma interaction *in vitro*. A. Human plasma plus radiogold. B. Human plasma plus gelatin and radiogold. Densitometric measurements of radioactivity made from radioautographs.

with and without added gelatin (2.5 mg/100 g of body weight) were given in one vein; blood samples (0.2 ml) were withdrawn from the opposite vein at various intervals using heparin coated syringes. Blood samples were centrifuged and the plasma was fractionated by paper electrophoresis. Paper electrophoresis was performed using a horizontal apparatus with the paper strips suspended by a central bridge. Four strips of Whatman 3 MM filter paper, 3.75 x 62 cm, were run simultaneously. Veronal buffer at pH 7.8 for rat plasma and pH 8.6 for human plasma and 0.05 ionic strength was used. After equilibration, 20 lambda of plasma was applied by streaking across a ruled line. A constant voltage of 100 volts was employed for 16 hours with a current of 4-5 milliamperes. After drying at 120°C for 30 minutes either radioautographs were made of the paper strips or the radioactivity on the strips was counted by a gas-flow chromatograph scanner driven by a synchronous mechanical feed from an Esterline-Augus recording apparatus. Thus the radioactivity patterns could be directly superimposed on the plasma protein patterns. The paper strips were then stained with bromphenol blue. Densitometry of the paper strips and radioautographs was performed with a Photovolt Model 501A instrument with optical density recorded at 2 mm intervals.

Results. When Aurcoloid alone was added to human plasma *in vitro*, the radioactive material did not migrate but remained at the origin. However, if gelatin and Aurcoloid were added to plasma the radiogold now had a mobility which corresponded with the alpha 2 globulin fraction (Fig. 1). Similar experiments using rat plasma gave the same results.

In the *in vivo* experiments the effect of gelatin was similar to that seen in the *in vitro* studies. Paper electrophoresis of plasma obtained from animals previously injected with gelatin and Aurcoloid demonstrated the similar location of the radioactive material and the alpha globulin fraction, whereas the radioactive gold did not migrate when Aurcoloid alone was injected (Fig. 2). The rate of disappearance of the Aurcoloid from the blood was markedly retarded in the gelatin injected animals (compare A and B, Fig. 2).

The relative mobilities of gelatin and the alpha globulin fractions of rat plasma were demonstrated by paper electrophoresis. Samples containing various combinations of Aurcoloid, gelatin and plasma were placed on filter paper and run simultaneously in the electrophoresis chamber. Aurcoloid alone, or in plasma, did not migrate. When Aurcoloid was added to gelatin the radiogold migrated with the gelatin whereas the combination of Aurcoloid, gelatin and plasma resulted in a

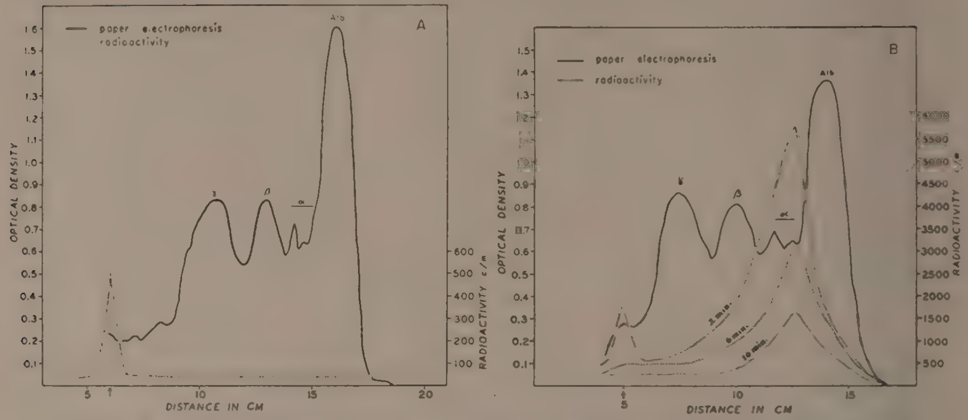

FIG. 2. Gelatin-plasma interaction *in vivo*. A. Blood sample removed 2 min. after radiogold injection. B. Blood samples removed at intervals after radiogold plus gelatin injection. Total radioactivity injected was similar in A and B.

still greater mobility of the radiogold which corresponded with the alpha globulin fraction (Fig. 3). These experiments suggested interaction between gelatin and the alpha globulin fraction and the possibility that radiogold particles are merely incorporated in the gelatin mass. The formation of a radiogold-gelatin-alpha globulin complex was associated with retention of radiogold in the circulation for longer intervals than was the case when interaction could not be demonstrated.

Discussion. Binding of radiogold colloid to the alpha and beta globulin fractions of human plasma, as demonstrated by Simon (2), may be explained by the fact that commercial radiogold preparations are stabilized by gelatin. The evidence presented in this investigation suggests that gelatin rather than

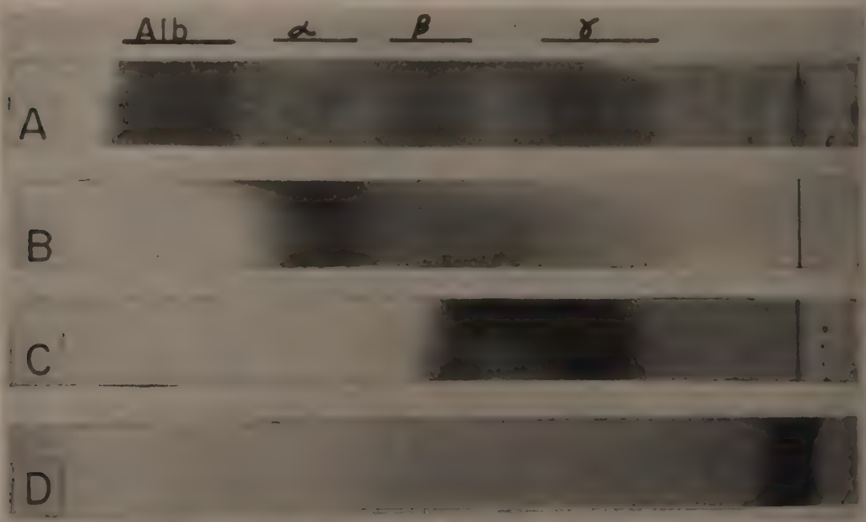

FIG. 3. Comparative electrophoretic mobilities of radiogold colloid. A. Electrophoretic pattern of rat plasma. B. Autograph: radiogold plus gelatin in plasma. C. Autograph: radiogold in gelatin. D. Autograph: radiogold in plasma.

metallic gold was bound by the alpha globulins. Although radiogold salts have been shown(3) to interact with the albumin fraction of plasma, Aurcoloid is relatively free of ionic gold.

The effect of gelatin in prolonging the retention of Aurcoloid in the circulation was apparently not mediated at the cellular level since colloidal gold was rapidly ingested by the RES regardless of whether the gold particles were coated with gelatin or plasma proteins(4). As Aurcoloid was eventually removed from the circulation by the RES, either the gold-gelatin-alpha globulin interaction was reversible or the total complex was phagocytised. Dissociation of the complex was not demonstrated in plasma electrophoretic patterns of animals injected with gelatin and Aurcoloid.

Summary. The relative mobilities of Aurcoloid and gelatin have been demonstrated *in vivo* and *in vitro* by paper electrophoretic methods. Addition of Aurcoloid and gelatin to plasma resulted in a radiogold-gelatin complex which migrated at the same rate as alpha globulins. Aurcoloid in plasma did not migrate. The formation of an Aurcoloid-gelatin complex was associated with a decreased rate of disappearance of radiogold from the circulation.

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Action of Parathyroid Extracts on Stable Bone Mineral Using Radiocalcium as Tracer.* (22229)

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When radiocalcium is injected into rats it becomes deposited in the skeletons of the animals in 2 forms which differ greatly in relative ease of mobilization and excretion. These forms of incorporation of radiocalcium and the parts of the skeletal bone mineral associated with each are referred to as the stable bone mineral and labile bone mineral(1,2). Fifty-two days after injection, radiocalcium is distributed almost exclusively in the stable fraction.

The present work describes the action of subcutaneously injected parathyroid extracts on radiocalcium (Ca^{45}) incorporated in the stable bone mineral.

Procedure. Twenty-four adult-male rats of comparable age and weight (380-420 g) were given intraperitoneal injections of high specific activity radiocalcium. This was administered in physiological saline solution in 3 doses of 2 cc each, allowing 2 hour intervals between injections. The total injected dose amounted to approximately 9.9×10^5 counts per minute according to the methods of sample preparation and counting conditions employed. After 72 days the left foreleg of each rat was amputated at the shoulder. Within this time the radiocalcium had presumably been distributed preponderantly in the stable fraction of the skeleton(1,2). The humeri of the amputated legs were divested of adhering flesh and stored in a freezer to be compared with opposite pairs from the same animal obtained later at the time of sacrifice. One day after removal of the legs the rats were given a subcutaneous injection of 100 USP units of

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Parathormone.[‡] One group of rats received only a single injection and were sacrificed in pairs at intervals of 2, 4, 8, 14, and 24 hours after injection. The remaining rats received daily injections and were sacrificed in pairs at the end of 2, 3, 4, 5, 7, 9, and 13 days. The humeri of the right legs were obtained and then the ends of all humeri, including those from the amputated legs, were separated from the shafts by means of a rotating dental Carborundum disk, using recognizable anatomical landmarks. Epiphyseal and diaphyseal regions were then analyzed for Ca^{45} in order to detect any intra-skeletal shift of radiocalcium which might have occurred subsequent to the injection of Parathormone. Total calcium was also determined. Urine was collected daily from 6 of the rats housed in metabolism cages, and from 2 control rats. The control animals had undergone amputation but received isotonic saline injections instead of Parathormone. Radiocalcium was determined in the urine and humeri, using the method of sample preparation described by Armstrong and Schubert(3).

Results. The results of radioactivity measurements of the individual bone samples are recorded in Table I. Analysis for total calcium in these samples showed no differences either on a dry fat-extracted or ash basis. It is therefore convenient to express specific activity in terms of ash weight. It was found that no large replacement of radiocalcium in the stable bone mineral by non-radioactive calcium occurred, since the specific activities of experimental samples and the opposite leg control samples were practically identical in each case. The epiphyseal and diaphyseal regions of a given pair of bones were not significantly different with respect to specific activity either before or after Parathormone treatment, demonstrating that no major intrahumeral shift of radiocalcium occurred.

TABLE I. Comparison of Ca^{45} in Epiphyseal and Diaphyseal Regions of Rat Humeri before and after Parathormone Injections, 23 Rats.

	Specific activity (% of injected dose/g of bone ash)			
	Epiphysis		Diaphysis	
Treatment	Control leg	Exp. leg	Control leg	Exp. leg
PTH,* single inj.				
2 hr	.66	.65	.71	.73
	.48	.45	.58	.59
4	.69	.66	.69	.70
	.57	.58	.62	.66
8	.61	.69	.69	.71
	.65	.64	.70	.69
14	.66	.64	.56	.62
	.62	.63	.69	.69
24	.56	.61	.65	.65
	.57	.53	.60	.59
PTH,* daily inj.				
2 days	.59	.56	.59	.60
	.63	.60	.65	.51
3	.61	.59	.65	.63
	.48	.48	.52	.51
4	.60	.57	.60	.43
	.67	.64	.66	.64
5	.57	.56	.61	.60
	.57	.59	.59	.60
7	.55	.54	.59	.55
	.55	.58	.57	.59
9	.65	.64	.64	.67
	.54	.59	.58	.59
13	.67	.62	.70	.71

* PTH = 100 USP units of Lilly Parathormone, inj. subcut.

There was, however, a greatly increased movement of radiocalcium out of the skeleton as a result of stable bone mineral resorption associated with the large dosages of Parathormone. Fig. 1 shows the effect of Parathormone treatment on the daily renal excretion of radiocalcium released from stable bone mineral. In order to improve counting conditions, the daily urine volumes of 3 rats were pooled, by housing the 3 together in the same metabolism cage. Group A in Fig. 1 refers to a group of 3 rats receiving daily Parathormone injections, the results being expressed as percent of the injected dose excreted per rat per day. Group B refers to a parallel, entirely similar group of 3 rats receiving Parathormone daily, the urine volumes from which were also pooled for counting. The control value is based on the urinary analysis of 2 rats, with amputated legs, receiving subcutaneous saline injections instead of Parathormone. Prior to treatment of the experimental rats with Parathormone and in the control rats, the urinary excretion

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[‡] A gift of Eli Lilly Co., Indianapolis, Ind.

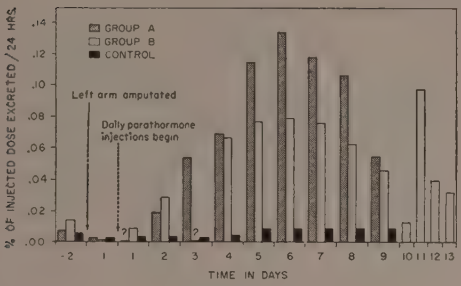

FIG. 1.

of Ca^{45} was between .009% and .013% of the injected dose per day. After a single Parathormone injection, radiocalcium in the urine was increased only slightly, but continued to increase during the first week of injections to a level of 0.08-0.14% (A). With repeated injections after the 6th day, however, the percentage of the injected dose in the urine began to decrease[§] and after 13 days was down to .04%. In general this decline reflects the loss of Parathormone potency usually observed after repeated injections of bovine parathyroid extracts in normal rats(4).

Discussion. No decreases in the total calcium content or specific activity of radiocalcium in the experimental legs as compared to their previously amputated counterparts were observed. This might merely indicate that Parathormone injections mediate a complete withdrawal of the materials comprising bone, not acting upon calcium independently. It is possible, however, that the amount of material mobilized from the humerus, compared to the large amount present, is so small as to be undetectable by direct analysis of the bone. It is also possible, though unlikely, that radiocalcium appearing in the urine is derived to a lesser degree from the humerus than from other bones of the skeleton.

Unfortunately separation of the epiphyseal from the diaphyseal regions of the humeri, for the purpose of detecting intra-osteal shifts of Ca^{45}, resulted in variable losses of material. An accurate comparison between bone weights of amputated legs and experimental legs was thus prevented, but it would seem likely that the small amount of mineral mobilized from the vast skeletal reserves would not be detectable even to this method. The urinary excretion of radiocalcium appears to be the most sensitive indicator of a Parathormone effect on Ca^{45} deposited in the stable bone mineral.

The increased quantity of radiocalcium appearing in the urine shortly after initiation of daily injections of Parathormone indicates that these extracts influence the stable bone mineral to yield calcium to the body fluids. During 13 days of Parathormone treatment in rats, at least 60 times as much Ca^{45} was lost by renal excretion as in control animals.

The present experiments have an interesting correlation to a theory of parathyroid action recently formulated by McLean, who describes a dual mechanism for controlling the level of calcium in the plasma(5). One part, independent of the parathyroids, acts by simple chemical equilibrium with the labile fraction of the bone mineral to maintain a constant but low plasma calcium level of approximately 3.5 meq/l. The second part of the dual mechanism is required to raise the plasma calcium to the normal level of 5 meq/l and is mediated by the parathyroid glands. McLean postulates that the parathyroid hormone releases calcium from the stable crystals of hydroxyapatite, as well as from the labile fractions of bone mineral. The present work provides experimental evidence that the stable bone mineral does indeed participate in releasing calcium to the body fluids as a result of injecting parathyroid extracts. This mechanism makes an otherwise inaccessible source of calcium available for homeostatic regulation.

Summary. The effect of Parathormone injections causing mobilization of radiocalcium from incarceration in stable bone mineral of rat, has been studied by observing specific activities of bones and amounts of radiocalcium appearing in urine prior to and following subcutaneous injections of bovine parathyroid extracts. From the greatly increased renal excretion of Ca^{45} following injection of

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[§] The unusually high value for Group B on day 11 was probably due to inadequate washing of cage and hence incomplete collection of urine on day 10.

parathyroid extracts it can be reasonably concluded that these extracts are able to produce mobilization of stable bone mineral from the rat skeleton.

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Incorporation of C^{14}-Acetate into Intestinal Fatty Acids of Rats with Cannulated Bile Ducts.* (22230)

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Incorporation of C^{14} from acetate into intestinal fatty acids has been demonstrated in experiments of short duration by various investigators(1-3). Evidence is available which indicates that the labeled intestinal fatty acids are synthesized independently of the liver fatty acids(2) and probably by the intestine itself(3,4). However, the possibility of transport of significant amounts of highly radioactive fatty acids from liver to intestine via the bile has not been investigated. This pathway was suggested to us by the findings of Gould *et al.*(5), that significant amounts of synthesized liver cholesterol are transported via the bile. The experiments described in this paper were designed to ascertain the role of bile in the acquisition of labeled long chain fatty acids by the intestine after administration of radioactive acetate.

Methods and materials. Adult male Sprague-Dawley rats weighing 200 to 300 g were used, and animals of the same weight were paired in each experiment. The bile duct of one rat was cannulated with polyethylene-50 tubing just distal to its bifurcation, and the cannula led to the outside of the body through an incision immediately below the right costal margin. A sham operation was performed in the control animal. Infection was not a problem and recovery usually occurred in 7 to 9 days. The sham-operated animal was pair-fed with the cannulated rat. During the first 2 days post-operatively there was low food consumption which led to a 5-10% decline in body weight. Subsequently, food consumption returned to normal and body weight remained at a constant level or showed a slight gain. When the rats had recovered, they were injected intraperitoneally with a tracer dose of radioactive acetate (1 ml of solution containing 3 mg of $CH_3C^{14}OONa$ and 10 μc of activity) and placed in a cage designed to hold animals immobile(6). Water was given *ad libitum* but food was withheld after C^{14} administration. Three pairs of animals were allowed to metabolize the radioactive acetate for 5 hours; 2 pairs were killed 30 minutes after injection. Samples of bile were collected in the 5-hour experiments at 30-minute intervals for the first hour and at hourly intervals thereafter. A 0.025 ml aliquot of each bile sample was dried on a planchet and counted as such to determine the relative activity of the whole bile. In the 30-minute experiments 0.025 ml samples were taken at 5-, 15-, and 25-minute intervals. No corrections for self-absorption were applied to these measurements. The liver, intestine, bile and combined contents of cecum and intestine were taken for chemical and

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TABLE I. Specific and Total Activity of Intestinal Fatty Acids of Cannulated Rats and of Sham-Operated Controls after Administration of $CH_3C^{14}OONa$.

Time killed after inj. of C^{14}-acetate	Specific activity, c/m/mg fatty acid		Total activity, c/m/100 g rat	
	Cannulated	Control	Cannulated	Control
5 hr	109	50	3600	4600
5 "	160	109	8500	4100
5 "	73	50	4600	3500
30 min.	61	49	4700	3200
30 "	52	21	4350	1750

radioactivity analyses of the total fatty acids. Chemical analyses were done as described previously(7). To each sample of bile was added 10 mg of inert palmitic acid before saponification that adequate material might be available for analysis. Correction for this addition was made in both chemical and radioactivity analyses. For determination of radioactivity fatty acids were oxidized to carbon dioxide by the method of Thorn and Shu(8) and samples counted as barium carbonate to a total of 3000 counts in windowless flow counter.

Results. The data presented in Table I show that the total activities of intestinal fatty acids of cannulated rats were similar to those of sham-operated controls. In fact, the values obtained in cannulated animals were in most cases slightly higher than those of controls. The specific activity of intestinal fatty acids was definitely higher in the cannulated group.

Analyses of biliary fatty acids revealed that specific activity values reached a maximum in one-half to one hour after injection and remained almost constant or declined slowly thereafter. The average specific activity and total activity of biliary fatty acids for 3 cannulated rats are shown in Table II. It can be seen that in each case total activity obtained in biliary fatty acids represented only 6 to 16% of that found in the intestine itself.

TABLE II. Radioactivity of Biliary and of Intestinal Fatty Acids of Cannulated Rats after C^{14}-Administration.

Rat No.	Specific activity, c/m/mg fatty acid		Total activity, c/m	
	Biliary fatty acids	Intestinal fatty acids	Biliary fatty acids	Intestinal fatty acids
1	93	109	1600	9950
3	53	160	400	15500
5	97	72	900	12800

The specific activity of whole bile reached a maximum in one hour or less but declined at a faster rate than that of the biliary fatty acids.

The amount and radioactivity of fatty acids of combined intestinal and cecal contents were compared for normal and cannulated rats (Table III). In 4 of the 5 pairs studied the fecal material from cannulated animals contained more fatty acid than did that from controls. In 3 of the 4 experiments in which data are available the specific activity of fecal fatty acids from cannulated rats was lower than that from controls. However, total activity of the fecal fatty acids was similar for the two groups.

There was no significant effect of deviation of bile on either specific or total activity of the liver fatty acids.

Discussion. These experiments indicate that bile does not transport significant amounts of liver-biosynthesized fatty acids to the intestine. This knowledge is important in studying the possible sources of highly labeled intestinal fatty acids observed after administration of C^{14}-acetate, and the findings are consistent with the hypothesis that intestinal synthesis from acetate is the source of labeled long chain fatty acids.

A greater amount of fatty acid was found in the fecal material of cannulated rats than was found in controls. Mild steatorrhea had been observed in cannulated rats 4 to 5 days postoperatively. The increased fecal fat content was no doubt due partly to incomplete absorption of ingested fat. However, increased secretion of endogenous fat in bile-cannulated animals has also been reported (9). The lower specific activity of fecal fatty acids of cannulated rats is consistent with the presence of more fat in the intestinal lumen acting to dilute labeled fatty acids synthesized from radioactive acetate. It should be expected that increased secretion of endogenous fat in the cannulated animals would

TABLE III. Radioactivity of Fatty Acids of Combined Intestinal and Cecal Contents of Cannulated and Sham-Operated Rats after Intraperitoneal Injection of C^{14}-acetate.

Time killed after inj. of C^{14}-acetate	Total fatty acids: Amt of fatty acids, mg		Specific activity, c/m/mg fatty acid		Total activity, c/m	
	C*	S†	C	S	C	S
5 hr	39.0	102.0	45.6	30.2	1780	3070
5 "	74.8	17.6	13.7	21.0	1025	370
5 "	53.0	22.6	11.8	20.3	625	460
30 min.	31.1	11.8	7.3	12.0	227	142
30 "	45.5	17.6	6.0	—	272	—

* C = Cannulated rat. † S = Sham-operated rat.

also result in increased total radioactivity in the fecal fatty acids. This was observed in 3 experiments but the opposite result was obtained in a fourth experiment.

The intestinal fatty acids of cannulated rats had a higher specific activity than those of sham-operated controls. The reason for this difference is not known.

Summary. 1. Deprivation of bile flow to the rat intestine by means of cannulation of the bile duct did not affect the ability of the intestine to accumulate normal amounts of highly labeled fatty acids after administration of C^{14}-acetate as shown by comparison with sham-operated, pair-fed controls. 2. Total radioactivity in the biliary fatty acids amounted to only 5-16% of the amount found in the intestinal fatty acids in the time periods studied. 3. Fatty acids of combined intestinal and cecal contents of cannulated animals contained more fatty acid but of a lower specific activity than those of control rats.

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Additional Serotypes of the APC Virus Group. (22231)

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Six serotypes of the adenoidal-pharyngeal-conjunctival (APC) group of viruses have been described(1,2). The purpose of this communication is to report eight additional serotypes from both human and simian sources which have been isolated in this and other laboratories.

Methods for characterization and identification of APC viruses. For purposes of classification, the cardinal attribute of the APC virus group is the group specific complement fixing antigen. This antigen is not shared with other known viruses, and complement fixing antibody against it is not produced by other known infections(1-3). The procedure presently employed for determining the presence of this antigen in candidate strains is as follows: using the complement fixation procedure previously described(2), the titer of the candidate antigen is determined by testing with a pool of human convalescent serums having high titer complement fixing antibody to the group antigen. Four units of the candidate antigen is then used in tests for antibody in paired serums of at least 4 persons, each known to have been infected with a different APC type, and each showing at least an 8-fold rise against the virus antigen

TABLE I. Neutralizing Antibody Titers of APC Prototype Rabbit Antiserums Tested against Prototype Viruses.

Rabbit antiserum	Virus types													
	1	2	3	4	5	6	7	8	9	10	11	D.C.	S.V.$_1$	Bertha
Type 1	40	0*	0	0	0	0	0	0	0	0	0	0	0	0
2	0	40	0	0	0	0	0	0	0	0	0	0	<20†	0
3	0	0	>160	0	0	0	0	0	0	0	0	<20	"	0
4	0	0	0	320	0	0	5‡	0	0	0	0	"	"	0
5	0	0	0	0	160	0	0	0	0	0	0	0	"	0
6	0	0	5‡	0	0	80	0	0	<20	0	0	<10	"	0
7	<20	<20	<20	<20	<20	<20	320	0	"	0	0	<20	"	0
8	0	0	0	0	0	0	0	>80	"	0	0	"	0	0
9	0	0	0	0	0	0	0	0	80	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	160	0	0	0	0
11	0	0	0	0	0	0	5	0	0	0	80	0	0	0
D.C.	0	0	0	0	0	0	0	0	<10	0	0	>160	0	0
S.V.$_1$	0	0	0	0	0	0	0	0	"	0	0	<20	>40	0
Bertha	0	0	0	0	0	0	0	0	"	0	0	"	0	>320

* 0 = <5. † Lowest dilution tested. ‡ Cross neutralization not observed with all rabbit serums.

of the type responsible for infection. When at least 3 of the 4 test serums show a fourfold or greater rise to the antigens of the candidate virus, the new agent can be regarded as almost certainly a representative of the APC group. Viruses are identified by the neutralization test (procedure 2) described previously(2). New serotypes are established when in reciprocal neutralizing antibody titrations with hyperimmune rabbit antiserums, the unclassified APC virus is found to be serologically distinct from all existing types. Each new prototype is examined for the presence of other properties of APC viruses, including resistance to ether, characteristic cytopathogenicity for HeLa cell cultures, trypsinized monkey kidney cultures, and explant cultures of human embryo epithelium, and lack of pathogenicity for suckling mice, adult mice, and rabbits inoculated by several routes.

Results. Fourteen serotypes have been delineated to date. Table I shows results of representative cross neutralization tests with the prototype strains. Very little cross neutralization was evident; the only heterologous neutralizations were low level, one directional crossing between Types 3 and 6, Types 7 and 11, and Types 4 and 7. Although not expressed in Table I, partial delay in cytopathogenic effects by low dilutions of heterologous antiserums was also occasionally observed(2).

The sources of the prototype strains are recorded in Table II. Three distinct types have been placed in an "incertae sedis" category. The D.C. strain grows so slowly in tissue culture that suitable comparisons with other prototype strains can be made only with great difficulty. The S.V.$_1$ and Bertha strains have not been isolated from human sources and possibly should be listed as a separate simian series.

All types are ether resistant, share the group complement fixing antigen, and are nonpathogenic for suckling mice, adult mice, and rabbits. All produce similar cytopathogenic changes in cultures of monkey kidney, human embryo epithelium, and HeLa cells. In HeLa cells growth of each type results in production of excess acid in the culture fluids.

Several of the newly established types demonstrated minor differences from the previously described biological attributes of the APC virus group(2). The cytopathogenic changes produced in HeLa cell cultures by

TABLE II. Prototype Strains of APC Group.

Type	Strain designation	Isolated from	Diagnosis	Isolated by
1	Ad. 71	adenoid	hypertrophied tonsils & adenoids	NIH(2)
2	Ad. 6	"	*Idem*	"
3	G.B.	nasal washing	common cold volunteer	"
4	RI-67	throat washing	primary atypical pneumonia	Hilleman & Werner(3)
5	Ad. 75	adenoid	hypertrophied tonsils & adenoids	NIH(2)
6	Ton. 99	tonsil	*Idem*	"
7	Gomen	throat washing	pharyngitis	Berge(5)
8	Trim.	eye swab	epidemic keratoconjunctivitis	Jawetz(4)
9	Hicks	stool	arthritis, rheumatoid ?, myelitis ?	Kibrick(6)
10	J.J.	eye swab	conjunctivitis	NIH(7)
11	Slobitski	stool	paralytic polio (Type 1 polio also recovered)	Kibrick(6)
"Incertae sedis"	D.C.	anal swab	Niemann-Pick disease ?	NIH(7)
"	S.V.$_1$	cynomolgous kidney	monkey kidney tissue culture	Hull(8)
"	Bertha	stool	chimpanzee with mild URI	Sabin & NIH(7)

Type 9, Type 10, and S.V.$_1$ were characterized by discrete round cells throughout the cell sheet, without clumping. The S.V.$_1$ strain, while cytopathogenic on primary passage, could not be maintained in serial passage in HeLa cell cultures, although it passed well in monkey kidney cultures. Large inocula of the D.C. strain were required to produce even abnormally delayed and slowly progressing cytopathogenic effects.

Several additional strains were not neutralized by antiserums to the fourteen prototypes, and these await completion of reciprocal tests before their qualifications as additional serotypes can be evaluated.

Summary. Fourteen serologically distinct APC virus types have now been established. Twelve types were recovered from human, and 2 from simian materials.

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Similarity of Glomerular Ultraviolet Absorptions in Diabetes Mellitus and After Cortisone Therapy. (22232)

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In the 20 years since Kimmelstiel and Wilson(1) described intercapillary glomerulosclerosis histologically, it has become a commonly recognized entity. Practically always, nodular glomerulosclerosis is a complication of diabetes mellitus. It is clearly not a subtype of arteriosclerosis or arteriolar nephrosclerosis(2), but little is known of the basis of its development. Experimental glomerular lesions closely simulating intercapillary glomerulosclerosis were described by Mann, Goddard and Adams(3) in rats rendered diabetic with alloxan, after 3 to 6 months, and by Rich and associates(4) in rabbits treated with 7.5 mg of cortisone daily. The pathologic process in rats was described as beginning with glomerular capillary erosions or increased deposits of periodic-acid-Schiff positive material in their walls and progressing to hyalin ball formation with concentric reticulin proliferations. Cortisone produced severe hyperglycemia and lipemia in rabbits. Recent reports by Becker *et al.*(5), Bloodworth and Hamwi(6), Wilens and Stumpf (7) confirm that cortisone therapy will lead to production of intercapillary glomerulosclerosis in normal or alloxan treated rabbits. Other factors such as vit. B_{12} deficiency, increased and altered blood lipids, and fat emboli have been reported as involved. It is uncertain how far the experimental lesions correspond to those of human diabetics, and to what extent cortisone hypersecretion might be present in human cases of glomerulosclerosis. Gordon(8) has reported unusually high levels of glycogenic adrenal steroid excretion and very low levels of 17-ketosteroid excretion in human cases of pathologically confirmed intercapillary glomerulosclerosis.

During an investigation of the histochemical and ultraviolet absorptive characteristics of kidney tissues from hypertensive patients, findings relevant to the hormonal background of diabetic glomerular lesions were unexpectedly encountered, and form the basis of this report.

Materials and methods. Freshly obtained kidney tissues from biopsies or autopsies were prepared by freeze-drying and embedded *in vacuo* in paraffin or polyethylene glycol (carbowax) with apparatus and methods previously reported(9). Microtome sections were cut at 4 μ and mounted on Vicor slides. Glycerin mounting with paraffin ringed Vicor cover slips was employed, with technics originally designed to prevent a loss of soluble substances prior to autoradiography. Ultraviolet microscopy with Polaroid color-translating instrument, was carried out in wavelength range 235-280 mμ as previously described(10). Enzyme treatment *in vitro* employed hyaluronidase in 1% solution, incubated 37°C for 30 minutes, and the solution washed off with distilled water. Treatment with trypsin, lecithinase, streptokinase and fibrinolysin was also employed, but without any significant results in the present problem. Following ultraviolet microscopy, the same sections were stained with Pearse periodic-acid-Schiff method originally introduced for staining pituitary cells(11), and studied for morphologic or histochemical abnormalities.

Results. Among hypertensive patients routinely investigated was a woman 38 years old with Cushing's syndrome, who had kidney biopsies made at the time of bilateral adrenalectomy. Each adrenal gland weighed about 40 g and was mostly composed of multiple cortical adenomas. She was not diabetic or glycosuric, and fasting blood sugar values

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TABLE I. Cortisone Therapy of 5 Cases Investigated by Ultraviolet Microscopy.

Sex	Age	Disease	Dose,[†] mg/day	Duration, days
♂	16	Osteogenic sarcoma	200, p.o.,	30
♂	19	Hodgkin's disease	300, "	9
♀[*]	38	Postop. Cushing's syndrome	40, i.m.	42
♀	42	Ulcerative colitis	300, "	6
♂	42	Pulmonary fibrosis	100, p.o.	16
			75, "	2
			50, "	2
			25, "	3

[*] Negative observations after bilateral adrenalectomy; positive findings before operation.
[†] p.o. = per os; i.m. = intramuscular.

were 82, 115, 120 and 143 mg %. Ultraviolet absorptions of glomeruli and arterioles from this patient were color-translated as orange in the shortest set of wave-lengths, 248, 240 and 235 $m\mu$, previously termed set 3.[‡] This abnormality of absorption had been observed before only in diabetes mellitus(9). Three additional cases of Cushing's syndrome available for study had been previously chemically fixed in Zenker's or formalin solutions, and they failed to show any abnormality of ultraviolet absorption comparable to that of frozen-dried tissues.

Tissues prepared by freeze-drying were available from autopsies of 4 other non-diabetic persons who had had cortisone therapy until death. On testing the ultraviolet absorptive properties of their glomeruli, the same abnormal orange color of their glomerular stroma, at short ultraviolet wave-lengths, was observed. Dosages of cortisone and basic clinical information are given in Table I. The original patient with Cushing's syndrome mentioned above was maintained postoperatively on 40 mg of cortisone daily until death 42 days later. At autopsy, kidney tissues obtained and tested as previously described failed to demonstrate the abnormality observed in biopsies. This was thought to indicate that over 50 mg cortisone per day was required to induce the abnormality of glomerular stromal absorptions.

Treatment *in vitro* with hyaluronidase of kidney sections removed the material responsible for abnormal absorption. After enzyme treatment, glomerular stromal absorptions appeared normal for the range 235-280 $m\mu$ investigated. It was considered that an abnormal mucopolysaccharide component, but not necessarily hyaluronic acid, had been affected by the hyaluronidase.

Hamster kidneys were also obtained from animals treated with cortisone, approximately 6 mg per week, for use as hosts of human cancer transplants(12). These tissues also showed the abnormal orange color of translated absorptions, and the responsible material was removed by hyaluronidase. It was not possible to determine whether the animals had been diabetic.

The localization of the abnormally absorptive material in the kidneys of patients treated with cortisone differed somewhat from that previously found in diabetics(9). Orange colors of the arteriolar walls in color-translated photomicrographs appeared after cortisone, but not in diabetes mellitus. The glomerular absorptive characteristics were indistinguishable. No similar absorptive abnormality was observed in the kidneys of several persons with essential hypertension or with hypertension secondary to pheochromocytoma, being concurrently investigated.

The renal architecture of kidneys from patients treated with cortisone was not definitely altered as judged by a scrutiny of periodic-acid-Schiff stained slides. The capillary walls stained positively with the PAS technic. Occasionally a dilatation was noted of glomerular capillaries near the glomerular roots. No ectasia of peripheral glomerular capillaries, glomerular capillary aneurysms, or thrombi were observed. No lesions were found simulating intercapillary glomerulosclerosis.

Discussion. Evidence has been presented, although indirect and perhaps tenuous, implicating cortisone as one factor essential for the development of human intercapillary glomerulosclerosis. Under the influence of cortisone administered in moderate dosages the glomerular stromal tissues were altered with the accumulation of an abnormal substance between the capillaries, and in their walls and those of arterioles. From the periodic-acid-Schiff staining of this material and its removal with hyaluronidase *in vitro* it was inferred to be a mucopolysaccharide complex, probably attached to protein, as usually occurs in ground substances and basement membranes.

[‡] Color translation by filters of 248 $m\mu$ as blue, 240 as green, and 235 as red was employed.

For the formation of nodular intercapillary glomerulosclerosis in the human kidney other factors are apparently necessary, the chief of which is a part of or accompanies diabetes mellitus. Whether this influence is attributable to hyperglycemia, general changes in the body mucopolysaccharides, hyperlipemia, fat emboli, or various steroid abnormalities in human diabetics is not evident from the present study. Additional information is anticipated from the delicate lipid staining possible with carbowax embedded frozen-dried tissues. Also it is hoped that a pathologic study of the endocrine glands from diabetics with glomerulosclerosis might show some other evidences of the cortisone hypersecretion postulated.

Summary. In the glomerular stroma and arterioles of non-diabetic persons treated with cortisone an abnormal substance was found with ultraviolet absorptive properties indistinguishable from those of the nodules of intercapillary glomerulosclerosis. Histochemical properties of the material suggest that it is a mucopolysaccharide complex. The evidence is considered that implicates cortisone as one factor in the development of human diabetic intercapillary glomerulosclerosis.

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Loss and Repair of Glucose-Disposal Mechanism in Dog Fed Fructose As Sole Dietary Carbohydrate.* (22233)

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Normal rats lose, to a considerable extent, their capacity to utilize glucose when they are fed for several days a diet containing 58% fructose as *sole* carbohydrate. The site of defective glucose utilization in the fructose-fed rats was localized by studying the metabolism of C^{14}-glucose, C^{14}-fructose, and C^{14}-acetate in various tissues of rats previously fed a diet containing either glucose or fructose as sole carbohydrate. The liver was the only tissue in which an altered metabolic pattern was observed after fructose feeding, and this alteration was manifested by a decreased capacity for converting glucose to glycogen, fatty acids, and CO_2. This altered metabolic pattern in the liver was apparently not the result of an insulin-lack, for insulin injections before the fructose-fed rats were sacrificed failed to restore the ability of their livers to utilize glucose(1).

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TABLE I. Dietary Feeding of Each Dog.

Diet fed	Days fed	Glucose-tolerance done on:	Glucose-tolerance curve in Fig. 1
A	First 7	A.M. of 8th day	I
B	8-15	16th	II
C	16-23	24th	III
B	One meal, P.M. of 24th day	25th	IV
B	Two meals during 25th day	26th	V
D	26-32	33rd	VI

Craig *et al.*(2) failed to observe, in man, an altered tolerance to glucose following the feeding of 250 g of fructose per day (for 3 days) as the sole dietary carbohydrate. It therefore became of interest to determine whether this response to fructose was limited to the rat and also whether another hexose, galactose, would also affect the liver's capacity for utilizing glucose. Experiments designed to test these two points are reported here.

Methods. Three adult male beagle dogs (9.8, 9.2, 11.6 kg) were maintained on Purina pellets before start of experiments. Experimental feeding periods were arranged consecutively so that each dog served as its own control. The general plan of the experiment is given in Table I, and the composition of experimental diets is given in Table II. The animals' tolerance to glucose was determined in the following manner. The last portion of a specified experimental diet (A, B, C, or D) was fed at 4 p. m. on the day before the glucose-tolerance test was carried out. At 9 a. m. on the following morning, initial blood samples were withdrawn from the femoral artery, and immediately thereafter the dogs were fed a test meal consisting of 4 g glucose/kilo body weight, mixed with a few grams of ground horse meat. At hourly intervals for the next 3 or 4 hours, blood samples were withdrawn from the femoral artery. Plasma reducing value was determined by the method of Somogyi(3) as modified by Nelson(4). Plasma fructose was measured by the method of Roe(5). Plasma galactose was estimated by the difference in reducing value before and after yeast fermentation.

TABLE II. Composition of Diets.

Constituent	Amt (g)/day/kilo body wt* Diet A	Diet B	Diet C	Diet D
Horse meat	47	35	35	35
Glucose	0	18	0	0
Fructose	0	0	18	0
Galactose	0	0	0	18

* Daily food allotment was divided into 2 equal portions; one portion fed at 9 a.m., the other at 4 p.m. In addition each dog received daily an adequate vitamin and salt supplement(6).

Results. Dogs fed a diet containing either no carbohydrate or glucose as sole carbohydrate. Results of the glucose-tolerance tests are given in Fig. 1. Each curve presents the average values obtained with 3 dogs. The dogs fed the diet containing no carbohydrate (diet A) for 7 days suffered the most severe loss in ability to dispose of exogenous glucose (curve I); the average plasma glucose values rose to 220 mg % 2 hours after administration of the glucose test meal and were still considerably higher than the initial level at 3 hours. In contrast, the average plasma glucose value after the dogs were fed the glucose-containing diet for 7 days reached a maximum of 120 mg % 1 hour after administration of the test glucose, and by the third hour had returned to the starting level (curve II).

Dogs fed fructose-containing diet. The average plasma glucose curve of dogs fed the diet containing fructose (diet C) for 7 days as the sole carbohydrate was similar to that obtained when the dogs were fed no carbohydrate (diet A); the highest plasma glucose value was 190 mg % at 2 hours, and the plasma glucose level was still elevated at the third hour after administration of the glucose test meal (curve III).

The feeding of one glucose-containing meal (diet B) after the 7-day period, in which fructose was the sole dietary carbohydrate, had no appreciable effect on the response of the plasma glucose to the glucose test meal, the average plasma glucose curve reaching a value of 180 mg % at 2 hours and 140 mg % at 3 hours (curve IV). However, after the third meal of diet B was fed, the average plasma glucose response was similar to that observed in the animals fed diet B for 7 days (curve V).

Dogs fed the galactose-containing diet. Feeding a diet containing galactose (diet D)

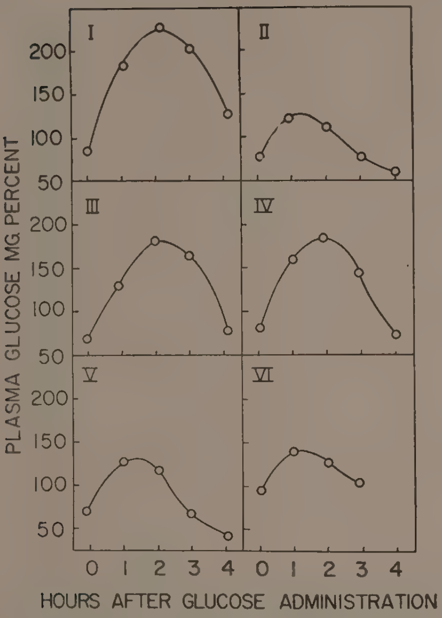

FIG. 1. Glucose tolerance tests of dogs after feeding of diets containing glucose, fructose, or galactose.

as the sole carbohydrate for 7 days produced a glycemic response to the glucose test meal similar to that observed after feeding diet B for 7 days (curve VI). In the galactose-fed dogs, the average plasma glucose level reached a maximum of 140 mg % 1 hour after administration of the glucose test meal, and returned to the initial level in 3 hours.

Summary. 1. Our earlier observation in the rat, that the feeding of fructose as sole carbohydrate results in a considerable loss in the animal's capacity to dispose of ingested glucose, is confirmed in another species, the dog. It is also shown that galactose is not the equal of fructose in bringing about impairment in glucose-disposal mechanism. 2. Feeding of a single meal containing 18 g of glucose/kg body weight failed to restore to normal the glucose-disposal mechanism once it was impaired by fructose feeding. A normal capacity for glucose disposal was observed after the dog had ingested 3 glucose-containing meals.

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Attempted Fractionation of Purified Bovine Growth Hormone.[*] (22234)

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By any of 3 isolative procedures(1-3) growth hormone (GH) can be obtained from bovine anterior pituitary glands as an apparently homogeneous protein of molecular weight about 47,000. The activity of such preparations does not depend on the presence of alanine and phenylalanine at the N-terminal ends of the 2 peptide chains(4), nor is the activity lost after partial digestion of the free-carboxyl end of the molecule with carboxypeptidase(5-7). It may be noted also that material with growth-promoting activity can, according to an unconfirmed claim (8), pass through a dialysis membrane.

This paper reports briefly an exploration of

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the possibility that a hypothetical "active constituent" might be leached out from the bovine protein GH preparation by fractional extraction with organic solvents. The results tend to support the view of Li and his colleagues(7,9-12) that the hormonal activity is a property of the bovine GH protein as isolated, and that, if the biological activity can be associated with material of lower molecular weight, this must be obtained by partial degradation of the parent molecule.

Methods. The bovine GH preparations were isolated by the method of Wilhelmi, Fishman and Russell(2), modified by conducting the initial extractions at pH 5.5 with 0.3 M KCl, and by collecting the crude GH fraction over the ethanol concentration range 10 to 30% (v/v), beginning at pH 8.5. The purified final products had essentially unit activity (10 μg/day injected for 10 days into 100 g hypophysectomized rats produced a gain in weight of 10 g). *Bioassays.* Growth-promoting potency was determined by the 10-day test(13) in 100 g male hypophysectomized rats, obtained from Hormone Assay, Inc., Chicago. In a series of 2-dose assays, the values obtained for the response (gain in weight) in g/$\log_{10}$ μg of hormone did not vary significantly, the mean value being 16.4 (with variance 0.75). Three to 5 animals were used for each dose of standard and unknown. The 10-day incremnts in width of the tibial epiphyseal cartilage were also observed routinely. These were variable, but roughly paralleled the weight increments. *Fractional extraction of bovine GH.* The usual procedure was to treat GH with ether-miscible mixtures consisting of a "non-polar" solvent admixed with a "polar" solvent in which GH was soluble (glacial acetic acid, pyridine, phenol, formic acid, or diethyl formamide, containing a small proportion of water). Most of the mixtures contained 5% (v/v) of water, but essentially similar results were obtained in a few experiments in which the solvents were almost anhydrous. Addition of thiodiglycol or phenol as antioxidant appeared to offer no advantage. Columns 5 or 7 mm in diameter and about 3 cm in height were set up with the slurry, obtained by addition of the "non-polar" solvent (or of a mixture rich in this solvent), at –10° or 0°C, to the dry-GH admixed with 10 parts of Supercel. In subsequent operations the temperature was usually kept below 2°C. The column was percolated with the solvent mixture at a restricted rate, the mixture being replaced progressively by one richer in the more "polar" constituent if addition of ether to the effluent did not yield or no longer yielded a precipitate. The precipitates obtained from the effluents by the addition of one volume of peroxide-free ether, followed if necessary by a trace of solid potassium acetate, were centrifuged, thoroughly washed with acetone, and finally taken up in water, made slightly alkaline with sodium or ammonium hydroxide, and lyophilized. The addition of ether to the effluents left in solution only a trace of nitrogenous material which was devoid of growth-promoting activity when tested after volatilization of the solvent at a low temperature—a procedure which substantially inactivated GH in control experiments. It was found, however that volatilization of the solvent system, ammoniacal *n*-propanol-water, caused little inactivation of GH. Accordingly, this isolative procedure was employed in fractional extractions performed with aqueous propanol systems.

In several instances, fractional extractions or acetone-dried or lyophilized powders of beef anterior pituitaries and whole pig pituitaries were carried out.

Results. Experiments on purified GH were conducted with 21 different solvent systems, and on dry gland powders with 4 systems. A representative sample of potency determinations of some of the more active fractions is presented in Table I. None of the products obtained in any experiment was significantly more active than purified bovine GH. Fractional extraction typically gave poor recoveries of activity, which was usually spread over several fractions. The behavior of acetone-dried or lyophilized gland powders was similar to that of purified GH. (The ethyl-acetate-formic acid system used with the beef gland powder was particularly effective in furnishing active fractions free of pigments, these being removed in the initial stages of

TABLE I. Representative Fractional Extractions.†

Exp. No.	Material in column	Components of solvent mixture*	% (v/v) of first component	Effluent vol (ml)	Yield of product (%)	Growth potency relative to purified GH
		With 5% (v/v) water				
20	Beef ant. pituitary, acetone powder, 4 g	Acetone-acetic acid	90	74	0	—
			80	57	.5	.4
			80-70	69	9.8	.5
			60-50	81	2.3	.5
			40-30	50	4.0	.3
61	Pig pituitary acetone powder, 3 g	Ethyl-acetate-formic acid	90	115	0	—
			85	120	1.9	.1 *
			78	100	3.0	.05*
			70	70	5.4	.2 *
			63	18	6.4	.1 *
55a	Purified GH, 80 mg, with Supercell	*Idem*	90	12	10	.3
			78-65	42	0	—
			60	26	12	1.4
			55	29	33	.9
			50	15	5	.5
62a	*Idem*	Methyl-formate-formic acid	90	20	1	.2
			78	14	0	—
			70	13	5	.6
			65	21	40	.5

* Adrenals enlarged (ca. 50%) at end of test.

† Other systems used were as follows ("non-polar" constituent in italics): *ether*-phenol; -acetone-phenol-water (containing ammonia); -water (containing hydrochloric acid); -ethyl-acetate; -pyridine; -phenol-dichloroacetic acid (aqueous, 0.2%); *ethanol* (containing ammonia); *n-propanol* (containing ammonia); -phenol-ammonium formate; -ammonium flavianate; -dichloroacetic acid (aqueous, 1%); *ethyl acetate*-acetic acid; -phenol; -pyridine; *dioxane*-phenol; -pyridine. All mixtures contained water, 5%, v/v.

the extraction.) Different runs with the same system varied unpredictably in the relative yields of material obtained from successive effluents, in the ease with which the precipitates could be re-dissolved in dilute alkali, and in the stage at which flow of solvent was arrested because of gelation of the column. When successive fractions from a single run were separately re-run, characteristic differences in the extraction pattern were observed, but in no case was complete recovery of material or activity achieved. Active material was extracted from purified GH only with acidic systems. No material was extracted when the "polar" constituent was diethylformamide (with or without dichloroacetic acid), or pyridine, although GH dissolved in pyridine could be recovered in good yield and activity by precipitation with acetone. No advantage resulted from the use of GH-flavianate in place of GH itself, or from the use of ether-immiscible solvent mixtures in which the "polar" constituent was water with additions such as ammonia, ammonia and urea, ammonium formate, flavianic acid or hydrochloric acid. Finally it may be mentioned that brief dialysis of GH against a solution of 6 M urea in glycine buffer, pH 9.5, or hydrochloric acid, pH 2.0 or 3.3, gave no evidence of dialyzable activity; the remaining protein had always lost some activity. No activity was found in the "extract" obtained by treating GH with liquid ammonia.

The possibility was considered that an "active constituent" separated from GH-protein might, if it were a much smaller molecule, show poor apparent activity in the bioassay because of excessively rapid absorption from the injection site, or because of rapid excretion. In contrast with ACTH, GH is inactive when injected as a suspension in beeswax-arachis oil, but aqueous suspensions (pH 8) adsorbed on aluminum phosphate were found to be as active as aqueous solutions. In no case was the activity of fractions obtained from GH found to be enhanced by the use of aluminum phosphate.

Discussion. The experiments with dry

pituitary gland powders indicate that fractional extraction with organic solvents would offer no advantages over present methods of extracting growth hormone. In one of the best experiments, Exp. 20 (Table I), the yield of active material, 30 to 50% as potent as the purified GH used as a standard, was 34 g/kg of fresh anterior lobes, representing very likely all of the growth-promoting activity originally present. The difficulties are that the fractionation cannot be repeated consistently, and that the products are poorly soluble and are likely to prove intractable to further purification. The pig pituitary fractions were evidently rich in ACTH, as indicated by adrenal enlargement in all of the test animals. An accurate estimate of the growth-promoting properties of these pig fractions is therefore impossible to obtain. Even if these fractions were relatively rich in growth-promoting activity, the indicated order of contamination with ACTH is a decided obstacle to further purification.

It was not possible to show that an "active constituent" could be extracted from purified bovine GH preparations. Such active fractions as were obtained—by procedures which invariably depressed the solubility and inactivated the bulk of the starting material—were essentially similar to the original GH by several criteria, most significantly perhaps in being poorly soluble in water in the pH range 6-7. No differences from the original GH were evident on examination of a few of the fractions for amino-acid composition (by paper chromatography of hydrolysates), for amide nitrogen content, or for the nature and content of free amino groups (by the fluorodinitrobenzene method). It would appear, therefore, allowing the reservation that the bioassay might falsely underestimate a highly active constituent of low molecular weight, that, in agreement with Pierce(14) the biological activity is a property of the protein as isolated, and as defined by the carefully detailed studies of Li *et al.*(7,9-12).

Summary. Purified bovine growth hormone, extracted fractionally with a wide variety of organic solvent mixtures, does not yield more active constituents than the protein starting material, nor do the products differ from it in other properties.

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Intracellular Localization of Enzymes in *Mycobacterium tuberculosis* var *Hominis*.* (22235)

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Millman and Youmans have shown that crude cell free extracts of *Mycobacterium tuberculosis* var. *hominis* H37Ra, oxidized lactic, pyruvic, acetic, fumaric, malic, succinic, citric, oxalacetic, and α ketoglutaric acids(1). This indicated that this strain of *M. tuberculosis* possessed a tricarboxylic acid terminal respiratory cycle. Millman and Youmans isolated a particulate red fraction from this extract by centrifugation at low speeds(2). This fraction, containing pleomorphic particles, oxidized fumaric, succinic, malic, oxalsuccinic, oxalacetic, α ketoglutaric, lactic, citric, and isocitric acids. A clear supernatant fraction, obtained by centrifuging the crude supernatant at 144000 x G, oxidized fumaric, malic, α ketoglutaric, lactic, and isocitric acids only in the presence of methylene blue as a hydrogen acceptor. The fact that the particulate red fraction did not require methylene blue for oxidation to occur led them to conclude that this fraction might contain a complete cytochrome system. This together with the fact that these particles reduced neotetrazolium suggested that these were identical with the structures which Mudd has called mitochondria(3). Yamamura isolated a particulate fraction from cell extracts of *Mycobacterium avium* by differential centrifugation(4). This fraction was composed of particles from 50-100 mμ diameter and was stained with Janus Green B. It was found to contain malic dehydrogenase and 3 cytochromes with bands at 554 mμ, 654 mμ and 595 mμ respectively. The author stated that this fraction behaved as a functional unit comparable to the mitochondria in animal tissue. Millman reported the isolation of a high speed sedimenting fraction composed of particles approximately 50 mμ in diameter(5). The 50 mμ particles contained some Krebs' cycle enzymes which were active in the absence of methylene blue and were cyanide sensitive. This fraction proved of greater interest when it was reported by Youmans *et al.* that it was capable of immunizing mice against challenge with a virulent strain of *Mycobacterium tuberculosis* var. *hominis*(6). Alexander and Wilson have isolated a large particulate fraction and several fractions containing smaller particles by differential centrifugation of cell-free extracts of *Azobacter vinelandii*(7). By quantitative studies they found that DPNH oxidase and p-phylenediamine oxidase occurred only in the granules, while the enzymes of the citric acid cycle were not exclusively localized in them. Isocitric dehydrogenase, α ketoglutaric dehydrogenase and aconitase were found quantitatively in the non-particulate cytoplasm. They concluded that only the particulate fractions contained an electron transport system to oxygen. In addition they found that succinic dehydrogenase and malic oxidase were totally in the particulate matter.

It is the purpose of this study to apply the methods first outlined by Alexander and Wilson(7) and Alexander(8) to a quantitative study of the distribution of enzymes in *Mycobacterium tuberculosis* var. *hominis* H37Ra.

Materials and methods. The work was carried out with the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* which was maintained by frequent transfers on the surface of modified Proskauer and Beck Medium(9). Organisms were grown as surface pellicles on modified P & B medium contained in 4 liter diphtheria toxin bottles. After 25 to 35 days of incubation at 37°C the pellicles were harvested by filtration through coarse sintered glass and washed with distilled water. Approximately 25 g (dry weight) of cells were obtained from 9 bottles. The slightly moist washed cells were then made into a

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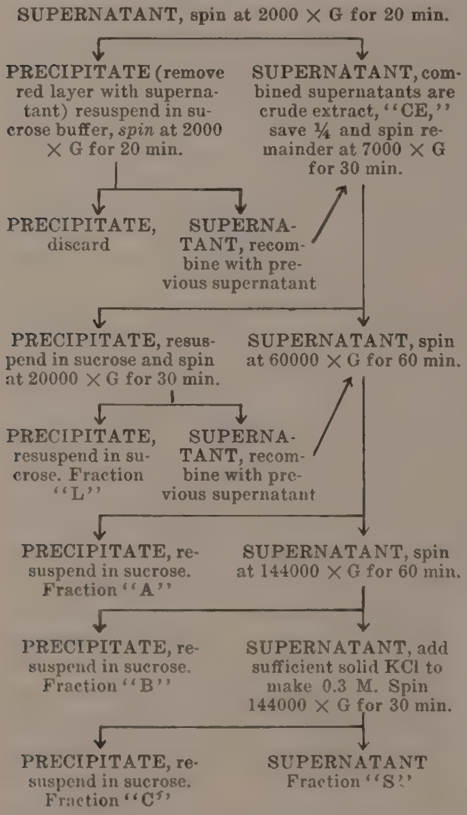
† Medical Research Fellow, National Foundation for Infantile Paralysis.

thick paste with 0.25 M sucrose dissolved in 0.01 M phosphate buffer pH 7.2 and were ground with powdered glass in a ball mill at 4°C for 18 hours. After grinding the cell mass was centrifuged at 2,000 rpm for 5 min. in an unrefrigerated International Centrifuge to remove intact cells and glass. The crude supernatant was fractionated as follows:


SUPERNATANT, spin at 2000 × G for 20 min.
PRECIPITATE (remove red layer with supernatant) resuspend in sucrose buffer, *spin* at 2000 × G for 20 min.
SUPERNATANT, combined supernatants are crude extract, "CE," save ¼ and spin remainder at 7000 × G for 30 min.
PRECIPITATE, discard
SUPERNATANT, recombine with previous supernatant
PRECIPITATE, resuspend in sucrose and spin at 20000 × G for 30 min.
SUPERNATANT, spin at 60000 × G for 60 min.
PRECIPITATE, resuspend in sucrose. Fraction "L"
SUPERNATANT, recombine with previous supernatant
PRECIPITATE, resuspend in sucrose. Fraction "A"
SUPERNATANT, spin at 144000 × G for 60 min.
PRECIPITATE, resuspend in sucrose. Fraction "B"
SUPERNATANT, add sufficient solid KCl to make 0.3 M. Spin 144000 × G for 30 min.
PRECIPITATE, resuspend in sucrose. Fraction "C"
SUPERNATANT Fraction "S"

All precipitates were homogenized with a hand driven glass homogenizer to insure a homogeneous suspension.

Enzymatic activity. Aconitase, fumarase, malic and succinic dehydrogenases were tested by the methods described by Colowick and Kaplan(10); catalase by the method of Lawrence and Halvorson(11); DPNH oxidase by the method of Green *et al.*(12); isocitric dehydrogenase by the method of Hogeboom and Schneider(13) and α ketoglutaric dehydrogenase by the method of Lindstrom (14). Cytochrome oxidase was determined in the following way. To the main compartment of Warburg reaction vessels were added 60 μmoles of p-phenylenediamine, 16 μmoles of histidine buffer pH 7.2, 5.0 μmoles of $MgSO_4$ and enzyme preparation. To the center well of each reaction vessel were added 0.2 ml of 10% KOH. The final volume was adjusted to 2.2 ml with distilled water and oxygen uptake measured at 37°C. One unit of enzyme is that amount causing an uptake of one μl oxygen in one hour. Oxygen uptake by control flasks (containing no substrate) was subtracted from the values obtained with the test flasks before the units were calculated.

Results. Fig. 1 to 4 show electron micrographs of the particulate fractions L, A, B, and C respectively. The preparations were shadowed with chromium to help make the particles electron stable as well as aid in visualization. The L fraction is made up mainly of 100-200 mμ particles. This fraction is probably equivalent to the particulate red fraction described by Millman(5). The A fraction contains particles 50 mμ in diameter. These are probably the same as described by Millman(5) and Youmans *et al.*(6). The B and C fractions contain particles not previously described by these authors. The B fraction contains particles 25 mμ in diameter. The C fraction appears to contain particles of approximately the same dimensions as the B fraction. On close inspection, however, these particles appear as aggregates of smaller units. Because of this it has been difficult to determine the size of an individual particle. However, if this fraction is equivalent to the one reported by Alexander(8) it should contain particles approximately 19 mμ in diameter.

In Table I are summarized the results of the enzymatic localization tests. Better than 79% recovery was obtained with all but two enzymes, namely DPNH oxidase and catalase. Of the enzymes giving high recovery, aconitase, fumarase, α ketoglutaric dehydrogenase, isocitric dehydrogenase, and malic dehydrogenase were found almost exclusively in the supernatant fraction. Cytochrome oxi-

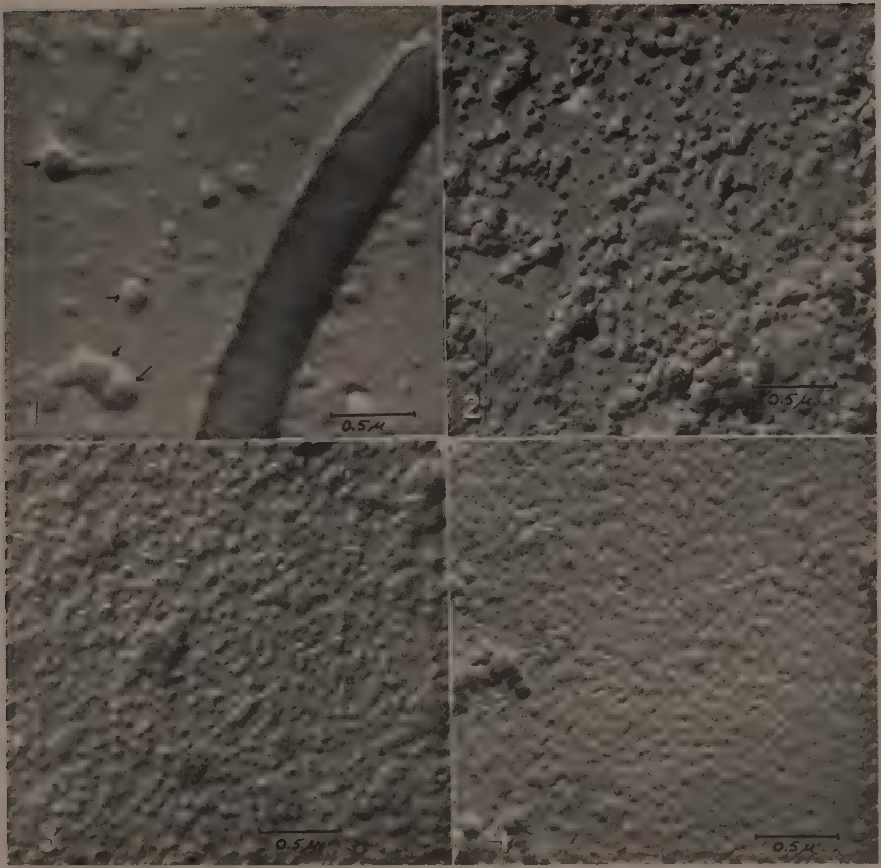


FIG. 1. Fraction L, shadowed with chromium. Contains intact tubercle bacillus and particles 100-200 mμ in diameter. × 30500.

FIG. 2. Fraction A shadowed with chromium. Contains particles approximately 50 mμ in size. × 30500.

FIG. 3. Fraction B shadowed with chromium. Contains particles approximately 25 mμ in diameter with some aggregates. × 30500.

FIG. 4. Fraction C shadowed with chromium. Contains mostly aggregates. × 30500.

dase and succinic dehydrogenase on the other hand were found mainly in the particulate fractions. Of the less completely recovered enzymes, DPNH oxidase was located almost entirely in the particulate fraction, while catalase was found mainly in the supernatant fraction. Only one enzyme showed any considerable activity in either the B or C fraction. This enzyme, fumarase, had about 12% of its total activity located in the B fraction. In relative activity the A fraction paralleled the L fraction rather closely, except that the total amount of enzyme located in the A fraction was rather less. The particulate fractions were not tested for the presence of isocitric dehydrogenase and α ketoglutaric dehydrogenase since the supernatant fraction possessed the major portion of these activities. Succinic dehydrogenase, on the other hand, gave no measureable activity in either the B, C, or S fractions.

Discussion. Our data indicate that the enzymes of the tricarboxylic acid cycle, with the exception of succinic dehydrogenase, are

TABLE I. Units of Enzyme Activity of Fractions of *Mycobacterium tuberculosis* var. *hominis* H37Ra.

Enzyme	"CE"	"L"	% CE	"A"	% CE	"B"	% CE	"C"	% CE	"S"	% CE	% recovered
Aconitase	14602	748	5.1	496	3.4	190	1.3	308	2.1	9900	67.8	79.7
Catalase	372509	1255	.33	1030	.28	4278	1.15	2653	.71	230132	61.78	64.25
Cytochrome oxidase*	4742	1697	35.8	693	14.6	173	3.6	31	.6	1175	24.8	79.4
DPNH oxidase	24105	7360	30.5	3180	13.2	348	1.4	84	.3	1100	4.6	50.0
Fumarase	58528	2550	4.4	1600	2.7	6900	11.9	820	1.4	35526	60.7	81.1
α-Ketoglutaric dehydrogenase	5006									3973	79.4	79.4
Isocitric dehydrogenase	45264									59520	131.	131. †
Malic dehydrogenase	2136000	8400	.4	33600	1.5	16800	.8	19600	.9	1620000	75.8	79.5
Succinic dehydrogenase	76	53	69.7	14	18.6							88.3

* For definition of unit, see text.

† High recovery, over 100%, probably due to partial inhibition of enzyme in "CE" by some metabolite.

either located primarily in the soluble fraction of the tubercle bacillus or in solubilized portions of the cell fragments. The presence of DPNH oxidase and cytochrome oxidase in the particulate fractions confirms the results reported in previous papers, (Millman and Youmans(1); Millman (5); Alexander and Wilson(7); and Mudd(3)) that the particulate fractions contain a complete cytochrome system and are equivalent in this respect to mitochondria of higher forms. The only metabolic difference noted between bacterial mitochondria and those of higher forms is the absence of the tricarboxylic acid cycle enzymes in the mitochondria of the former. Our results with the enzymes of the tricarboxylic acid cycle differ only slightly from those obtained by Alexander and Wilson(7). They found malic dehydrogenase in the particulate fractions while we found it in the supernatant fraction. They also found marked enzymatic activity in the B and C fractions(8) while we found negligible activity in these two fractions.

The fact that the activity of fraction A paralleled that of fraction L suggests the possibility that either the L fraction may be made up of smaller particles, or that the A fraction, and possibly the B and C fractions, may have arisen from the breakdown of the L particles.

Summary. By grinding cells of *Mycobacterium tuberculosis* var. *hominis* H37Ra using a ball mill and by differential centrifugation four particulate fractions and one supernatant fraction were obtained. Aconitase, catalase, fumarase, α ketoglutaric dehydrogenase, isocitric dehydrogenase and malic dehydrogenase were located almost entirely in the supernatant fraction, while cytochrome oxidase, DPNH oxidase, and succinic dehydrogenase were found to be associated mainly with the two largest particulate fractions.

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New Technic for Study of Drug Actions on Bronchial Resistance in Isolated Lung.* (22236)

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In the estimation and evaluation of drug effects on specific organs and tissues, the use of isolated preparations perfused with a balanced salt solution has certain evident advantages. Drug and electrolyte concentration may be accurately and reproducibly controlled without the possible complications of metabolism, excretion, and binding by plasma proteins; temperature may be varied at will, and reflex and endocrine control is eliminated. Nevertheless, no completely satisfactory technic has been described for observation and measurement of drug actions on the bronchioles in an isolated lung. The object of this report is to describe an isolated lung preparation in which an attempt has been made to meet 4 basic requirements of *in vitro* technic: 1. Sensitivity to drugs should approach that shown in the intact animal. 2. Experimental conditions should simulate physiological conditions as closely as possible. 3. The method should not be encumbered with complicated apparatus and technic. 4. Results obtained should be easily interpreted and evaluated.

Most of the isolated lung preparations described in the literature have been designed for physiological studies, and technics used are rather elaborate(4-10). Usually the perfusion fluid was defibrinated or heparinised blood, which has the theoretical advantage of reducing oedema formation but introduces many new variables; it has been shown, for example, that substances may be liberated from blood which influence the tone of bronchioles(1,2). In cases where drugs were used, the sensitivity was 10-100 times lower than in the present method. Sollmon and von Oettingen(11) suggested perfusion of lungs with Locke's fluid through the bronchial tree. Although this method is very simple, it appears highly unphysiological and possesses very low sensitivity to drugs; moreover, the fluid must flow through the vascular system as well as the bronchioles, and flow rate will therefore depend on effects on the vasculature.

A large number of technics using intact animals have been described, some of which are based on principles similar to those used in the present method, *e.g.*,(12).

Method. General Principles. The object of this technic is to measure alterations in bronchial resistance in isolated lungs perfused through the pulmonary artery with a balanced salt solution. This is achieved by recording the pressure in a semiclosed system connected with the trachea while applying alternating pressure changes outside of the lungs. The recorded pressure changes will then be greatest when the bronchi and bronchioles are widely dilated, and smaller when the resistance to the passage of air between trachea and alveoli is increased. The apparatus is shown schematically in Fig. 1. It consists essentially of a warming chamber,

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* The opinions or assertions contained herein are private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

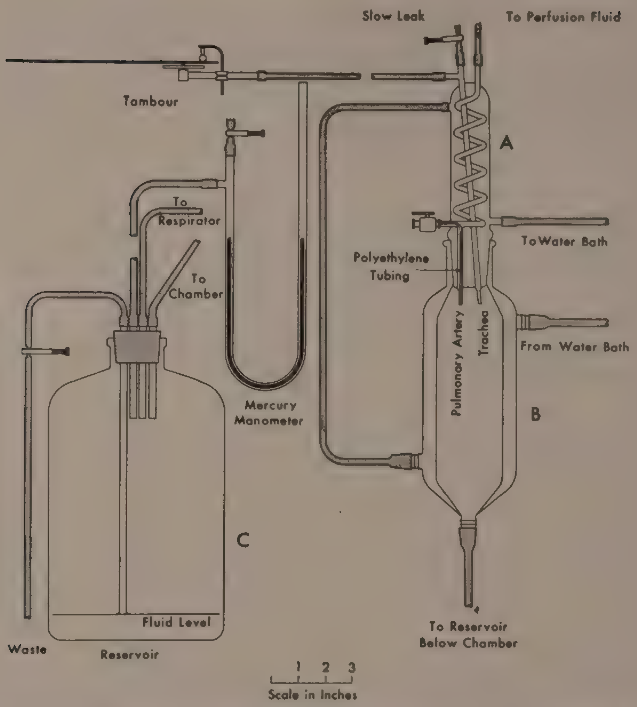
Slow Leak
To Perfusion Fluid
Tambour
A
To Respirator
To Chamber
Polyethylene Tubing
ToWater Bath
Pulmonary Artery
Trachea
From Water Bath
B
Mercury Manometer
C
Fluid Level
Waste
Reservoir
To Reservoir Below Chamber
1 2 3
Scale in Inches

FIG. 1. Semi-schematic diagram of apparatus for lung perfusion. A is a warming chamber for tidal air and perfusion fluids; B, the lung chamber and C, the drainage reservoir, which is actually situated immediately beneath B.

A, in which the perfusion fluid and tidal air are warmed by water circulated from a thermostatic water bath at 38°C; a water jacketed lung chamber, B, in which the lungs are suspended by cannulae in the trachea and pulmonary artery from the appropriate outlets from A; and a drainage vessel C, which serves the dual purpose of collecting the fluid draining from the open pulmonary veins, and acting as a buffer between respiration pump and lung chamber to minimize alterations in applied pressure resulting from changes in volume of the lungs. A is connected with B by means of an airtight ground glass joint, and C is located immediately beneath B, to which it is attached with rubber tubing. *Perfusion* fluid is delivered to A from a Marriotte bottle, the air inlet of which is at the level of the pulmonary artery so that no positive perfusion pressure is used. Flow of fluid is maintained by pumping action of the applied air pressure changes, and is approximately 3-5 ml/min. A sidearm from the perfusion tube near the lower end of the warming vessel is plugged with a rubber stopper through which a hypodermic needle is inserted. On the inside this is connected to a polyethylene tube leading to the opening of the pulmonary artery, and on the outside a stopcock is attached. This serves to allow injections into the perfusion fluid to be made with minimum dilution and maximum speed of effect, while the stopcock prevents an air leak when the syringe is not in place. The dead space of the injection system is 0.2 ml and all injections were therefore washed in with 0.5 ml. The fluid used was a modified Krebs solution as follows: $NaCl$, $NaHCO_3$, KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, glucose, epinephrine in concentrations of 692, 210, 16.2, 35.4, 14.7, 18.7, 100.0 and .01 mg/100 ml, respectively. The glucose was added immediately before use, and the solution was then equilibrated with 5% carbon dioxide in oxygen for at least 30 minutes. Epinephrine was added after oxygenation. The *tracheal cannula* is connected through the warming chamber with a Marey tambour recording on a kymograph. A sidearm bears a piece of rubber tubing with adjustable clamp, which during operation is kept almost closed. This slow leak serves to equalize the average pressure with atmospheric and improves the sensitivity to bronchoconstrictors. The *vessel C* is connected with the lung chamber B, respiration pump, mercury manometer and drainage tube. Total capacity of B and C is approximately 5 liters, and the respiration pump is set to move 600-800 ml of air in and out with each stroke. In a closed system the amplitude of the applied pressure change would therefore be 90-120 mm Hg. The drainage tube clamp is opened until this amplitude falls to 50-70 mm Hg, and this maintains the fluid level in C constant and at the same time equalizes the average pressure with atmospheric. Without the slow leaks on either side of the system to equalize the average pressure there was a tendency for the pressure to mount on one side and decline on the other, causing a progressive change in average volume of the lungs and in the sensitivity to drugs.

Procedure. Guinea pigs of both sexes weighed 450-550 g. The animal is given 400 units of heparin intramuscularly and 15 min. later is killed by a blow on the head. The carotid artery is immediately opened on one side, care being taken not to damage the trachea. A tracheal cannula is then inserted and artificial respiration started. The anterior wall of the thorax is opened and a polyethylene cannula tied into the common pulmonary artery through the right ventricle. The lungs are then dissected out with as little handling as possible and the heart removed. Artificial respiration is stopped and the 2 cannulae are attached to the appropriate outlets from the warming chamber A by means of adaptors. The water-jacketed vessel B is placed in position and the respiration pump started immediately. The useful life of this preparation is very dependent on the speed with which this procedure is carried out and degree of handling of the lungs. Five to 7 minutes were sufficient in our experiments.

Results. General Performance. When the preparation is first set up there ensues a progressive dilatation of the bronchioles, as evidenced by an increase in the size of the tracing, for 20-40 min. This process may be accelerated by small injections of epinephrine (2 γ), the effect of which tends to persist indefinitely (Fig. 2). During the first 60-90 min. of operation, the lungs gradually accumulate fluid until a steady state is reached, after which sensitivity to bronchoconstrictors remains approximately constant for the life of the preparation (6-8 hours). This accumulation of fluid does not interfere with movement of air, nor with response to drugs; on the contrary, the preparation does not become stable until it appears to be almost solid in the expired state.

In some preparations, however, fluid accumulated in the trachea and bronchi and persisted in spite of aspiration. This interfered grossly with movement of air and such preparations were useless for the study of drug actions. The causes of this phenomenon have not so far been determined with certainty, but certain precautions were found to reduce the incidence to a minimum and have been incorporated in the technic described. They are: 1. Addition of epinephrine to the perfusion fluid in a concentration of 1:10,000,000. In some experiments in which this was initially omitted, fluid accumulated in the trachea but recovery occurred when epinephrine was introduced and aspiration carried out. 2. Use of zero perfusion pressure. A wide range of perfusion pressures was tried, and in general the lower the pressure the less is the tendency to fluid accumulation in the trachea and bronchi. 3. Speed in dissection; avoidance of excessive handling of lungs and of inhalation of blood. Following a blow on the head, blood is readily aspirated, and rapid introduction of a tracheal cannula to avoid this is essential. 4. Premedication with heparin to prevent

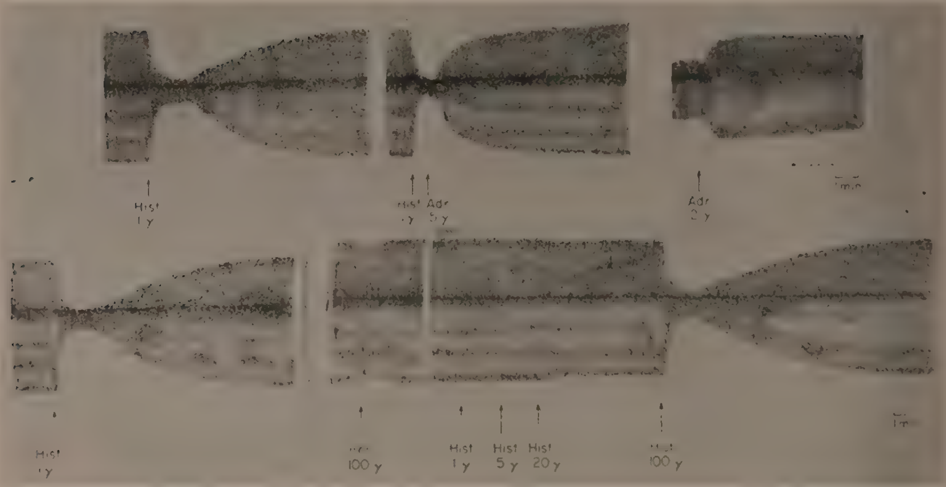

FIG. 2. Tracings showing effects of histamine, adrenaline and benadryl on the isolated lung. Tracings on top line were obtained from one preparation, those on bottom line from another. Effect of adrenaline alone is shown immediately after preparation was set up.

pulmonary emboli. 5. Positive pressure artificial respiration from the time spontaneous respiration stops until the preparation is finally set up. 6. Use of young animals. If the animal is too small, however, the tracing obtained is too small to work with; 450-500 g guinea pigs were found to be a suitable compromise.

Response to histamine. The injection of 1 γ histamine diphosphate produces a rapid reduction in amplitude of the tracing, indicating bronchoconstriction. The effect persists for a little over a minute and complete recovery ensues in 6-8 min. (Fig. 2, top line). Perfusion rate is almost unchanged, a 0-10% reduction in outflow being seen. This response is reproducible for several hours provided that the conditions are maintained constant. However, temporary mechanical disturbance of perfusion flow may cause a longer lasting change in sensitivity to histamine. It is therefore advisable for assay purposes to use a Marriotte bottle large enough to last for the entire experiment without refilling. An increase in sensitivity up to 10-fold may be achieved by omitting epinephrine from the perfusion fluid; however, there is then a greater tendency for fluid accumulation in the trachea, and recovery from histamine is slower. Sensitivity to histamine may be reduced very greatly by antihistamines. Fig. 2 (lower tracing) shows a sequence taken from an experiment in which a single injection of 100 γ of benadryl reduced the sensitivity to histamine by a factor of approximately 100.

Response to other agents. Acetylcholine produces a response closely resembling that of histamine, with slightly faster recovery, at

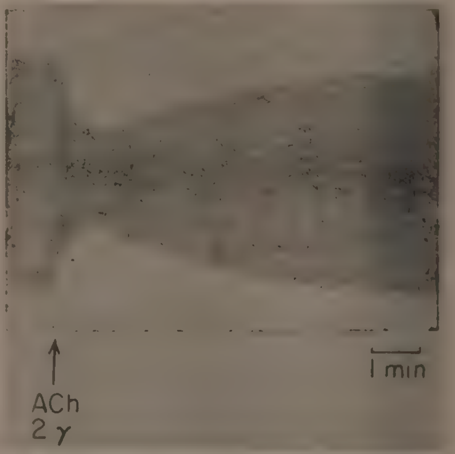

FIG. 3. Tracing showing effects of acetylcholine on isolated lung.

about 3 times the dose (Fig. 3). This response may be blocked by atropine. Morphine sulphate produces no bronchoconstriction in doses of up to 250 γ.

Bronchodilators have little or no action on the standard preparation, presumably because the bronchi are maintained maximally dilated by the epinephrine. Even epinephrine, in doses up to 5 γ, produces only a slight and very temporary effect. When superimposed on a typical response to histamine, however, epinephrine has a marked effect in accelerating recovery (Fig. 2); papaverine and theophylline also show some dilator activity in rather large doses (250 γ and 500 γ respectively). As mentioned earlier, epinephrine also produces a well marked effect on a preparation newly set up, and if epinephrine is omitted from the perfusion fluid, marked sensitivity is shown to bronchodilators.

Summary and Conclusions. A technic for the observation and measurement of the effects of drugs on bronchial resistance in an isolated perfused lung preparation is described. This preparation shows an absolute sensitivity to histamine comparable to commonly used biological assay objects, and shows a reproducibility adequate for assay purposes. Its simplicity makes it suitable also for teaching demonstrations of the effect of drugs on the bronchioles.

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Potentiation of Glycogenolytic Effect of Epinephrine by Desoxycorticosterone in Certain Skeletal Muscles.* (22237)

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The glycogenolytic effect of epinephrine on skeletal muscle (hind limb) is inhibited by cortisone acetate in normal rats(1), by adrenal cortical extract in adrenalectomized rats(2), and by growth hormone in hypophysectomized rats(3). On the other hand, adrenalectomy(2,4) or the injection of thyroxin(3,4) potentiates the glycogenolytic action of epinephrine and thyroidectomy depresses it(4). Those hormones which inhibit this effect of epinephrine also induce a storage of glycogen in these skeletal muscles (glycopexic effect)(1,2,3,5). While it is usually stated that desoxycorticosterone does not stimulate glycogen storage in muscle(5-8), there is no information on whether this mineral-corticoid influences epinephrine effects on carbohydrate metabolism in the intact animal. This report will show that desoxycorticosterone acetate (DOCA) potentiates the glycogenolytic action of epinephrine in certain skeletal muscles.

Materials and methods. Adult female rats of the Long-Evans strain were selected for uniformity of age and weight within each ex-

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* Aided by grant from Muscular Dystrophy Assn. of America, and Sage and Sackett Research Funds of Department of Zoology, Cornell University.

periment. The animals were fed a diet consisting of calf meal and dog pellets *ad libitum*. The adrenalectomized animals received 1% salt solution in addition to drinking water. Subcutaneous injections of 2 mg DOCA† per day in peanut oil were administered for 3 days prior to autopsy. Hormone treatment was begun one week after adrenalectomy and 3 weeks after thyroidectomy. Epinephrine was injected intraperitoneally in doses of 10 γ per 100 g body weight one hour prior to autopsy. In all experiments the rats were starved for 24 hours prior to the administration of epinephrine. The muscles used in this study were the *rectus femoris,* a portion of the abdominal muscles and the diaphragm. The method for anesthetizing the animals, removing the muscles, and determining glycogen has been previously described(9). The glycogen is reported as mg of glucose per 100 g of muscle, wet weight (averages with the standard error of the mean).

Results. The injection of DOCA into normal intact rats had no significant effect on the glycogen content of any of the muscles used in this study (Table I-A), which confirms earlier work(5-8). The injection of epinephrine into the normal rats decreased the glycogen content in all 3 muscles significantly. When the rats were pretreated with DOCA, however, epinephrine induced a greater loss of glycogen in the leg and abdominal muscles as compared to the loss observed in the untreated rats. There was no potentiation of the epinephrine effect in the diaphragm. If one compares the loss of glycogen expressed as a percentage of the respective initial levels in the DOCA-treated and control animals, it is noted that the greatest potentiation of epinephrine occurred in the leg muscle (44% *vs* 23% loss), with an intermediate response in the abdominal muscle (62% *vs* 46%).

In view of the previous finding that glucocorticoids inhibited the glycogenolytic action of epinephrine in some muscles(1,2), it was considered possible that the administration of DOCA suppressed the endogenous secretion of the gluco-corticoids via the pituitary(10) which would essentially remove an inhibitor to the action of epinephrine. To test this possibility, the effect of DOCA was studied in adrenalectomized rats.

The data in Table I-B show that adrenalectomy alone had no significant effect on the glycogen levels of the 3 muscles when compared to the intact controls. Following the administration of epinephrine, a significant decrease in muscle glycogen was exhibited by all 3 muscles. Furthermore, the loss of glycogen was greater in the adrenalectomized rats than in the intact rats but only in the leg muscle. Pretreatment of the adrenalectomized rats with DOCA caused a significant increase in glycogenolysis only in the leg muscle.

Collip *et al.*(4) and Leonard(3) have reported that thyroxin potentiates the glycogenolytic action of epinephrine in hypophysectomized animals. This finding introduces the possibility that the potentiating effect of DOCA might be conditioned by the thyroid hormone: accordingly, the effect of DOCA on glycogenolysis was investigated using thyroidectomized rats.

The data show (Table I-C) that thyroidectomy alone causes no appreciable decrease in muscle glycogen. Administration of epinephrine to the thyroidectomized rats caused no significant decrease in the glycogen levels of the *rectus femoris* or abdominal muscles; the diaphragm exhibited a significant decrease. Pretreatment of the thyroidectomized animals with DOCA failed to increase the glycogenolysis induced by epinephrine.

Progesterone is reported to have properties similar to DOCA in regard to salt and water retention(11) and also to its inability to stimulate the deposition of glycogen in muscle(12,13). Accordingly, an experiment was made to determine whether progesterone, like DOCA, would potentiate the glycogenolytic action of epinephrine.

The data in Table I-D show that intact rats pretreated with 2 mg progesterone per day for 3 days exhibited no significant increase in glycogenolysis above that obtained with epinephrine alone.

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† Desoxycorticosterone acetate and progesterone were donated by Schering Corp., Bloomfield, N. J.

TABLE I. Effect of DOCA and Progesterone on Epinephrine Induced Glycogenolysis.

Exp.	No. of rats	Glucose (mg/100 g muscle)		
		Rectus femoris	Abdominal	Diaphragm
		Part A—Intact rats		
Control	16	333 ± 13	428 ± 11	285 ± 7
DOCA treated	6	321 ± 15	424 ± 17	306 ± 11
E† treated	15	256 ± 6	233 ± 10	107 ± 8
E & DOCA treated	10	178 ± 9*	160 ± 14*	111 ± 9
		Part B—Adrenalectomized rats		
Control	10	339 ± 10	377 ± 12	313 ± 17
E treated	15	228 ± 5	203 ± 9	122 ± 8
E & DOCA treated	14	169 ± 9*	183 ± 10	96 ± 8
		Part C—Thyroidectomized rats		
Control	4	309 ± 27	377 ± 21	269 ± 13
E treated	12	293 ± 15	281 ± 26	122 ± 16
E & DOCA treated	12	269 ± 17	291 ± 22	132 ± 20
		Part D—Intact rats		
P† treated	5	352 ± 10	430 ± 13	246 ± 21
P & E treated	10	251 ± 9	198 ± 13	86 ± 21

* In all cases where DOCA increased glycogenolytic effect of epinephrine significantly ($P < .01$), results are marked with an *.

† E = Epinephrine; P = Progesterone.

Discussion. These results show that the mineral-corticoid, DOCA, in some manner enhances the glycogenolytic action of injected epinephrine in the *rectus femoris* and the abdominal muscles, but not in the diaphragm. In contrast, the gluco-corticoid, cortisone, inhibited this effect of epinephrine to the greatest extent in the leg muscle, less in the abdominal muscles and not at all in the diaphragm(1). There is a basic difference in sensitivity to epinephrine among these three muscles in the untreated rat; the diaphragm is the most sensitive and the *rectus femoris* the least.

The possibility that DOCA was repressing the secretion of the adrenal gluco-corticoids via pituitary ACTH, thus removing an inhibitor to the action of epinephrine, was discounted when it was observed that DOCA potentiated glycogenolysis in the adrenalectomized animal. This suggests that DOCA is acting directly on the muscle to potentiate epinephrine.

At first sight it appeared that some interrelationship with the thyroid gland was involved, since DOCA did not potentiate epinephrine in the thyroidectomized animal. However, the loss of thyroid activity in an animal is known to decrease the absorption of injected epinephrine(14) and this decrease could be of such magnitude that any effect of DOCA would be unobservable. It is reported that DOCA increases the absorption of electrolytes from the intestine in adrenalectomized rats(15) and increases the permeability of the skin(16). If DOCA, in potentiating epinephrine, acts by increasing the permeability of the target organ to the passage of epinephrine, adequate thyroid secretion may be essential for this effect. It seems more likely that thyroidectomy acts by preventing absorption of the epinephrine at the injection site.

A concept of mutual antagonism between the 2 types of adrenal cortical steroids on certain stress reactions and on the specificity of their antagonism to definite target organs has recently been discussed by Selye(17). It may be that the normal maintenance and regulation of glycogen storage in skeletal muscle is dependent in part on the relative endogenous production of the two types of adreno-corticoids and epinephrine. There is little evidence at present to indicate how much the normal output of epinephrine affects the level of muscle glycogen in the intact animal. Bowman(18) has shown that in the hypophysectomized animals which lack the glycopexic hormones, removal of the whole adrenal gland induces a rise in the glycogen level in fasting rats. The increase in muscle glycogen would seem to be caused by the elimination of endogenous epinephrine.

Finally, it was observed that progesterone was unable to potentiate the glycogenolytic action of epinephrine.

Summary. Desoxycorticosterone acetate increased the glycogenolytic action of epinephrine in the *rectus femoris* and abdominal muscles but not in the diaphragm in intact rats. In adrenalectomized rats, the effect was limited to the *rectus femoris*. Thyroidectomy decreased epinephrine induced glycogenolysis, and DOCA was unable to nullify this inhibi-

tory effect. Progesterone had no effect on the glycogenolytic action of epinephrine.

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Inhibition Studies with *Streptococcus faecalis*. Acridines and Acridones. (22238)

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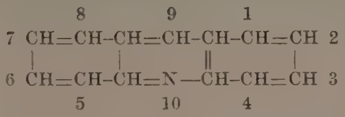
The investigations of Woods(1) led to the finding that the sulfonamides owed their chemotherapeutic activity to their property of interfering with the utilization of p-aminobenzoic acid by the microbial cell. This observation has stimulated the synthesis and testing of compounds related closely in structure to known metabolites for trial in the controlled interference with selected metabolic reactions. In the instance of folic acid, this has given rise to a series of folic acid blocking agents(2), some of which have proven usefulness(3), though limited, in a variety of ways. The interference with the utilization of folic acid was applied by Hitchings, among others, to develop compounds of biological interest. As a result Hitchings and his associates observed(4,5) that the rather active anti-folic, pyrimethamine, appeared also to possess some promise against the malarial parasite, though continued study may have modified this view somewhat(6,7).

In view of the findings that compounds which interfere with the utilization of folic acid may have application in other disorders for which agents are needed, a reinvestigation of 53 available acridines(8,9) and acridones (10), prepared initially for testing against the malarial parasite, was undertaken.

Methods. The folic acid assay medium utilized in this study was slightly modified from that of Teply and Elvehjem(11). The modifications consisted of omitting the xanthine and of utilizing "vitamin free" casein hydrolysate (Nutritional Biochemicals) in place of the charcoal treated hydrolysate originally suggested. When folic acid was added in amounts up to 1.0 mμg per ml of medium the acid production upon titration with N/10 alkali went up from blanks of about 0.5 ml of alkali per tube to 9.5 to 10 ml of alkali per tube at the higher levels of added folic acid. The test compounds were added to tubes containing 0.32 mμg of folic acid per ml of medium, a level designed to support half maximum growth as judged by acid production. This usually ranged about 4.5 to 5.0 ml of N/10 alkali per tube. For the assays, the test compounds were added to duplicate tubes usually at 4 levels of test: 1.6, 8,

TABLE I. Acridines Which Interfere with Utilization of Folic Acid by *S. faecalis*.

Compound,



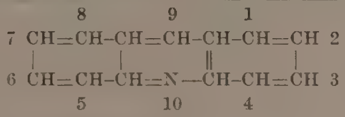
Substituent at 9	Derivatives	Activity vs. folic acid, $\mu g/ml$
$9\text{-}NH_2$ (aminacrine)	$4\text{-}CH_3$	8/ 40
$9\text{-}NH(CH_2)_3NHCH_2CH_2OH$	$2\text{-}OCH_3$	40/200
" $NHCH_2CHOHCH_3$	"	"
" $NH(CH_2)_2CHOHCH_3$	"	"
$9\text{-}NH(CH_2)_4N(CH_3)_2$	$2\text{-}OCH_3$ 6-Cl	"
" $N(C_2H_5)_2$	"	"
$9\text{-}SCH(CH_3)\ (CH_2)_3N(C_2H_5)_2$	"	"
$9\text{-}NH(CH_2)_3S(CH_2)_2N(C_2H_5)_2$	"	"
$9\text{-}NH\ C_6H_4N(CH_3)_2$	"	"

40, and 200 μg per ml of final medium. The tubes were plugged, sterilized about 12 minutes at 120°C, cooled at room temperature, and inoculated(12), with a drop of a suspension of *Streptococcus faecalis* (A.T.C.C. No. 8043). The growth of the organism was estimated titrimetrically after 72 hours using brom thymol blue as the indicator.

Results. The acridines and acridones tested and their ability to interfere with the utilization of limiting levels of folic acid in the medium are summarized in Tables I-III. To characterize the inhibitory activity of the compounds, in instances where inhibitory activity was observed, an arbitrary ratio term has been used. The upper (and lesser) figure represents the amount of test compound which exercised little or no inhibition of acid

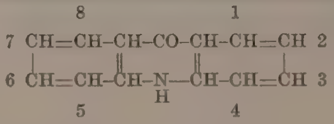
TABLE II. Acridines Which Do Not Interfere with Utilization of Folic Acid by *S. faecalis*.

Compound,



Substituent at 9	Derivatives
$9\text{-}NH_2$	$2\text{-}OCH_3$ 6-Cl, $3\text{-}NO_2$ $6,7\text{-}(OCH_2)_2$
$9\text{-}NHCH_2CH_2OH$	$3\text{-}NO_2$ $6,7\text{-}(OCH_2)_2$
$9\text{-}NHCH_2CH_2NH_2$	$2\text{-}OCH_3$ 6-Cl
$9\text{-}(3'\text{-}C_5H_4N)$	"
$9\text{-}(6'\text{-}OCH_3\text{-}8'\text{-quinolyl-}NH_2)$	"
$9\text{-}NH(CH_2)_2NH(CH_2)_2CH_2OH$	" (unsub.), $6\text{-}NO_2$ $2,3\text{-}(OCH_3)_2$
" $NHCH_2CHOHCH_3$	$2\text{-}OCH_3$, $2\text{-}OCH_3$ 6-Cl, $2,3\text{-}(OCH_3)_2$ 6-Cl
" $NHCH_2CHOHCH_2OH$	$2\text{-}OCH_3$, 6-Cl, $2\text{-}OCH_3$ 6-Cl
" $NHCH_2C(OH)\ (CH_3)_2$	$2\text{-}OCH_3$, $2\text{-}OCH_3$ 6-Cl
$9\text{-}S(CH_2)_2N(C_2H_5)_2$	$2\text{-}OCH_3$ 6-Cl
$9\text{-}O(CH_2)_2N(C_2H_5)_2$	"
$9\text{-}NH(CH_2)_3NHCH_2CH_2OH$	"
" $NHCH_2CHOH\ CH_3$	"
" $NH(CH_2)_2CHOH\ CH_3$	"
" $NHCH_2C(OH)\ (CH_3)_2$	$2,3\text{-}(OCH_3)_2$ $6\text{-}NO_2$
" $N(C_2H_5)CH_2CHOH$	$2\text{-}OCH_3$ 6-Cl
$9\text{-}NHCH_2CHOHCH_2N(C_2H_5)_2$	$2,3\text{-}(OCH_3)_2 6\text{-}NO_2$ (entozon)
$9\text{-}NHCH(CH_3)\ (CH_2)_3N(C_2H_5)_2$	2-OH 6-Cl, $2\text{-}OCH_3$ 6-Cl (quinacrine), $2,3\text{-}(OCH_3)_2$ 6-Cl, $2\text{-}OCH_3$ $6\text{-}CH_3$, 2-F 6-Cl
$9\text{-}NHC_6H_4N(CH_3)_2$	
$9\text{-}NHCH_2CH(C_6H_5)(CH_2)_2N(CH_3)_2$	$2\text{-}OCH_3$ 6-Cl
" $(CH_2)_2N(C_2H_5)_2$	"
$9\text{-}NHCH_2(C_6H_5)C_5H_8NCH_3$	"
" $C_5H_8N\ CH_2C_6H_5$	"

TABLE III. Effect of Acridones on Utilization of Folic Acid by *S. faecalis.*

Compound,  Substituent at 1	Derivatives	Activity vs. folic acid, μg/ml
$1\text{-NH(CH}_2)_2\text{N(C}_4\text{H}_9)_2$	4-CH_3, $4\text{-CH}_3 6\text{-Cl}$	None
$1\text{-NH(CH}_2)_3\text{N(C}_2\text{H}_5)_2$	3-Cl, $4,6\text{-Cl}_2$	8-40
″	4-Cl	40-200
$1\text{-NHCH}_2\text{CHOHCH}_2\text{N(C}_2\text{H}_5)_2$	$4,6\text{-Cl}_2$	″
$1\text{-NHCH(CH}_3)\ (\text{CH}_2)_3\text{N(C}_2\text{H}_5)_2$	4-Cl	″

production (growth) of the organism. The lower (and larger) value represents the amount of test substance which appreciably or completely inhibited acid production by the organism. This method of evaluating the test compounds has proven more reproducible than the calculation of the 50% molar inhibition index. The latter has been observed to vary to a disturbing extent following small changes in the test conditions, whereas the ratio term has remained constant in the face of minor modifications in the testing of the compounds.

The results summarized in Tables I-III probably may be rendered more meaningful if compared with those obtained under these conditions of test on some known effective anti-folics. The inhibition ratios against folic acid and citrovorum factor, respectively, were: aminopterin 0.008/0.04 and 0.2/1.0, amethopterin 0.00032/0.0016 and 0.04/0.2, and pyrimethamine 0.0016/0.008 and 25/100.

From the data of Tables I and III it may be seen that 40 to 200 μg of 10 acridines and of 5 acridones per ml of medium proved to be inhibitory. These amounts were 1000-fold (or more) those needed by the anti-folics given above. The acridine and acridone inhibitors thus appear to be primarily of theoretical interest at this time. The activities of the inhibitory acridines and the acridones were further differentiated. With the same organism and with 2.0 mμg of citrovorum factor(13) per ml of medium as the limiting nutrient 6-chloro-9(4-diethylamino-1 methylbutylthio)-2-methoxy acridine and 1-(3-diethylamino-2-hydroxypropylamino)-4, 6-dichloroacridone were no longer inhibitory. In the presence of 2.5 μg of thymine(14,15) per ml of medium and with *S. faecalis* as the test organism, 9-[3-(3-hydroxybutylamino)propylamino]-2-methoxyacridine no longer inhibited. In the presence of 2.5 μg of thymidine(16) and with *S. faecalis* as above, 2-methoxy-6-chloro-9 - (4′-dimethylaminophenylamino)-acridine, 6-chloro-2-methoxy-9-(4-dimethylaminobutylamino)acridine, and 9-[3-(2-hydroxyethylamino) propylamino] - 2-methoxyacridine no longer inhibited the organism.

The remaining 5 acridines of Table I (9-aminoacridine, 9-amino-4-methylacridine, 2-methoxy-6-chloro - 9(4 - diethylaminobutylamino)propylamino)-acridine, 9-[3-(2-hydroxypropylamino]-2-methoxy acridine, 6-chloro-2-methoxy-9(γ-(β'-diethylaminoethylthio)-propylamino) acridine, and 4 of the acridones of Table III (3-chloro-1-(3-diethylaminopropylamino)-acridone, 4-chloro-1-(3-diethylaminopropylamino)-acridone, 4, 6-dichloro-1 - (3-diethylaminopropylamino)-acridone, 4-chloro-1-(4-diethylamino-1-methylbutylamino)-acridone) which inhibited *S. faecalis* in the presence of limiting amounts of folic acid were inhibitory whichever of the above nutritiles were limiting in the medium. Whether the inhibitory activity of these compounds was due to interference in the metabolic sequence of nucleic acid metabolism or whether it was due to blocking some other metabolic sequence is not known at this time.

Another point of interest arises from these studies. It may be noted that the effective antimalarial, quinacrine (mepacrine), does not inhibit the organism in the presence of limiting amounts of folic acid. Parenthetically, a number of salts of quinacrine were tested so that the results are not limited to one salt. Thus, the acridine anti-malarials appear to owe their anti-malarial activity to some property other than interference in nucleic acid metabolism. This must be qualified, however, for the reason that it is not proven that results with *S. faecalis* may be carried over *in toto* to the malarial parasite.

Like quinacrine, the anti-bacterial Entozon (nitroacridine 3582, Hoechst) did not inhibit the test organism under the conditions of the test with folic acid as the limiting nutrilite. Contrariwise, the anti-bacterial 5-aminoacridine (aminacrine) inhibited the organism whichever nutrilite was present in limiting concentrations.

Thus, the studies with *S. faecalis* demonstrate interesting selectivity with various acridines and acridones. Whether they will remain of theoretical interest only remains to be determined.

Summary. 1. 46 Acridines and 7 acridones were tested against *Streptococcus faecalis* in the presence of limiting amounts of folic acid. It was observed that 10 of the acridines and 5 of the acridones inhibited the organism. However, the amounts of test compounds needed were 1000 times (or more) those needed by aminopterin, amethopterin, and pyrimethamine. 2. Further study demonstrated that inhibition exhibited by acridines and acridones could be overcome by citrovorum factor, thymine, or thymidine in some instances. The toxicity of 5 of the acridines and 4 of the acridones was not overcome by any of the nutrilites tested which suggests that their inhibition was not due to interference with the nucleic acid metabolism of the organism. 3. An additional observation was that the anti-malarial quinacrine and the anti-bacterial Entozon did not inhibit *S. faecalis* in the presence of limiting amounts of folic acid. The anti-bacterial 5-aminoacridine was inhibitory whether the limiting nutrilite was folic acid, citrovorum factor, thymine, or thymidine.

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B M R and Thyroxine Secretion Rate in Salivariectomized Female Rats.[*] (22239)

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The salivary glands have been known for many years to play a role in iodine metabolism by secreting (excreting?) blood iodide as a component of saliva. Recently a function of the salivary glands in iodine metabolism has been reported by Fawcett and Kirkwood(1,2) in the rat, namely to deiodinate diiodotyrosine and monoiodotyrosine, the latter by virtue of the presence of an enzyme, tyrosine iodinase. These workers also reported that the rapid deiodination of intravenously injected diiodotyrosine which occurs in normal rats was prevented (up to 60 minutes) in rats previously salivariectomized. Consequently the suggestion has been made (1) that not only is the blood level of thyroxine in the rat maintained by the rate of thyroxine synthesis in the thyroid gland but also by the rate of destruction of thyroxine and/or its precursors, monoiodotyrosine and diiodotyrosine wherever this may occur.

The purpose of the experiments reported herein was to determine the effect of salivariectomy on thyroid function as shown by the BMR and thyroxine secretion rate; *viz.* do the salivary glands play an essential role in extrathyroidal thyroxine metabolism or can their function be dispensed with without seriously disturbing thyroxine metabolism?

Procedure. Young adult Sprague-Dawley female rats, fed Purina Laboratory Chow and water *ad lib.*, were used in these 2 experiments. In the *first experiment,* 8 female rats were salivariectomized under pentabarbital (Nembutal) anesthesia and allowed to recover; 8 other normal females served as control animals. The individual body weight range for these 16 animals was 100-125 g. The BMR's of these rats were determined from O_2 consumption data obtained by using a closed, large glass desiccator with a water manometer and an attached 10 cc syringe filled with air. CO_2 was absorbed by NaOH (Ascarite). The pressure inside the desiccator was kept relatively constant by replacing periodically the oxygen consumed with air from the syringe. Constant temperature was maintained by use of a water bath at room temperature. The BMR's used in the final calculation for the individual rats were averages of at least 2 determinations. In the *second experiment,* thyroxine secretion rates of 23 salivariectomized and 25 intact rats were calculated by comparing thyroid gland weights at autopsy of a control group of rats having received no thiouracil with 3 groups of animals having received .1% thiouracil[†] in their drinking water for 2 weeks; in addition, 2 of the latter 3 groups were injected daily with 3 γ and 6 γ respectively of thyroxine[‡] subcutaneously. The thyroxine crystals were dissolved in distilled water with just enough 0.1 N NaOH added to make them soluble. The thyroxine solution was prepared weekly and stored in a refrigerator between injections. The body weight range of these animals was 150-180 g.

Results. The results of calculating the BMR's of normal rats and salivariectomized rats, 4-40 days postoperatively, are shown in

TABLE I. Comparison of BMR's of Salivariectomized and Intact Rats. Differences between experimental and normal groups are not statistically significant using the t-test.

		Mean BMR (Cal./M²/hr)		
		Days postoperative		
Rats	No.	4-6	18-20	38-40
Salivariectomized	8	44.4 ± 5.6	44.1 ± 6.2	40.1 ± 5.0
Normal	4	47.1 ± 6.9	46.6 ± 3.1	
"	4			41.2 ± 5.5

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[*] Department of Physiology and Pharmacology paper No. 103.

[†] Thanks are due Lederle Laboratories for a generous supply of thiouracil.

[‡] Crystalline thyroxine was supplied through the courtesy of G. S. Reed of the Squibb Institute for Medical Research. This material contained approximately 90% l-thyroxine and 10% d-thyroxine.

TABLE II. Data for Determination of Daily Thyroxine Secretion Rate in Rats. Differences in thyroid weights between respective normal and experimental groups are not statistically significant using the t-test.

Groups	Thiourea	Daily thyroxine dose, γ	No. animals	Mean initial body wt, g	Mean body wt at autopsy, g	Mean thyroid wt, mg %
Normal	No	0	7	161	183	6.08 ± .7
	Yes	0	6	166	185	15.4 ± 1.3
	"	3	6	159	175	10.7 ± 2.1
	"	6	6	164	182	7.2 ± .8
Experimental	No	0	5	156	180	6.14 ± .7
	Yes	0	6	153	169	16.6 ± 2.2
	"	3	6	153	172	9.78 ± 2.9
	"	6	6	151	167	5.9 ± 1.9

Table I. In spite of the fact that at the time of each determination the mean BMR of the control animals was higher than that of the experimental animals, this difference is not statistically significant when analysed with the t-test.

Experimental data used in determining the daily thyroxine secretion rate of salivariectomized and intact rats are shown in Table II. Differences between the corresponding normal and experimental groups receiving otherwise similar treatment are statistically insignificant when analyzed with the t-test. By plotting the thyroid gland weights from Table II against different groups of treated animals as shown in Fig. 1, an estimation of mean daily thyroxine secretion is obtained. For the control group of rats this value is approximately 6.6 γ thyroxine secreted per rat per day and for the experimental group, 5.7 γ/rat/day. Because l-thyroxine is the more active if not *the* active form of thyroxine(4), the true secretion rate of l-thyroxine would be 90% of these values or 5.9 γ and 5.1 γ/rat/day respectively. Converting these figures to a weight basis, the values become approximately 3.4 and 2.9 γ l-thyroxine secreted/100 g rat/day.

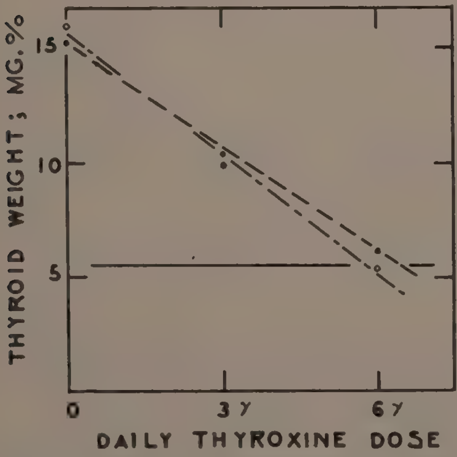

FIG. 1. Plotted data from which daily thyroxine secretion rate is calculated. ———, all rats receiving no thiouracil; – – – –, normal rats receiving thiouracil; —·—·—, salivariectomized rats receiving thiouracil.

Discussion. As a result of their experiments, Fawcett and Kirkwood(1) concluded that salivary glands of the rat are capable of acting as "thyroids-in-reverse" by degrading thyroxine into its constituent parts. This degradation by the salivary glands presumably occurs in steps, one of which(2) they have identified, namely, the deiodination of monoiodotyrosine under the control of tyrosine iodinase. This enzyme is reported to be comparatively abundant in the 3 salivary glands of the rat, particularly in the submaxillary and parotid glands. These 2 glands in the rat have greater concentrations of tyrosine iodinase than the thyroid gland where this enzyme catalyzes one step in thyroxine synthesis. In addition to the above action in the salivary glands, it has been reported that the normally rapid deiodination of intravenously injected diiodotyrosine does not occur in salivariectomized rats(1), at least within the space of 1 hour.

From the results of the present experiments it is concluded that the role of the rat's salivary glands in controlling thyroxine levels in the body by fostering the degradation of thyroxine and/or its breakdown products is not an indispensable role under normal circumstances because the salivary glands can be removed without causing a statistically significant change in thyroxine metabolism as revealed by the BMR and thyroxine secretion rate.

Perhaps after salivariectomy, control of thyroxine level through degradation is assumed by other tissues at a slower but none-the-less equally effective rate. The kidney and stomach have been reported to possess tyrosine iodinase in low concentrations(2). Evidence is accumulating to show that many tissues can deiodinate thyroxine with formation of triiodothyronine as for example the kidney in which the process appears to be controlled by an adaptive enzymatic mechanism(3).

On the other hand, an important point in determining the comprehensive role of the salivary glands in thyroxine metabolism still remains to be demonstrated; *i.e.*, their participation in the degradation of thyroxine itself to an extent relatively greater than that of other peripheral tissues which are metabolically stimulated by thyroxine and thus "utilize" thyroxine in their normal metabolism. If the salivary glands do not destroy thyroxine to a relatively greater extent than other tissues do, it is evident that a salivariectomy will probably not result in a recognizable change in BMR or thyroxine secretion rate.

Obviously more experimental data need to be available before an adequate appraisal of the normal role of the salivary glands in thyroxine metabolism can be made, however, in view of the present finding, it appears that this role is nonessential or adaptive.

Summary. Salivariectomy performed in young adult female rats produced no statistically significant effect on BMR or thyroxine secretion rate. It is concluded that rat salivary glands play a nonessential or adaptive role in thyroxine production and metabolism.

NOTE: After this paper was submitted for publication, two other papers(5,6) reported similar conclusions reached by different methods.

The technical assistance of Miss Gwendolyn Slover is gratefully acknowledged.

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Serological Response in Chickens to Beta-Propiolactone Treated Newcastle Disease Virus. (22240)

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In a previous study(1) it was demonstrated that Newcastle disease virus (NDV) was rendered noninfectious for chickens by treatment with 0.025% beta-propiolactone (BPL). The vaccinated birds developed no symptoms of Newcastle disease while 97.4% unvaccinated ones developed symptoms or died from the disease. Hemagglutination inhibition tests performed on paired sera from the vaccinated group showed a significant rise in titer.

The present communication presents additional information about the antibody response in the vaccinated group.

Materials and methods. White Leghorn chickens were used. All birds were from the same apparently disease-free flock. Beta-propiolactone was supplied by Dr. T. L. Gresham, B. F. Goodrich Research Center, Brecks-

TABLE I. Serological Response in Chickens to Newcastle Disease Virus Vaccine.

No. sera tested	Amt vaccine, ml	HI titer pre-vac,* avg	Post-vac, 16 day HI titer avg	No. sera tested	Pre-vac, serum index avg	Post-vac, serum index avg
13	.5	1:14	1:500	9	1.48	3.80
10	1.0	1:12	1:520	6	1.02	3.83
10	1.5	1:13	1:690	6	1.00	3.75
10	2.0	1:18	1:832	6	1.38	4.93
43	Avg	1:14	1:635	27	1.22	4.07

* vac = vaccination.

ville, Ohio. To facilitate dispensing of small volumes of BPL used, the BPL was diluted 1:10 with cold, sterile, distilled water and per cent concentration was based on undiluted BPL. In preliminary experiments, it was determined that treatment of NDV-infected allantoic fluid with 0.025% BPL for 2 hours at 37°C, destroyed infectivity of the virus for embryonated chicken eggs. It was also determined that heating NDV in allantoic fluid without added BPL for 2 hours at 37°C did not appreciably inactivate virus infectivity. Several serial embryonating egg passages were made with the virus by the allantoic route, prior to preparing a pooled allantoic fluid-virus suspension. The stock virus possessed a $10^{-9.5}$ LD_{50} titer when tested by injecting 0.1 ml of virus into embryonating eggs. All chickens in the experiment were bled from the heart to obtain pre-vaccination blood samples and stored in a frozen state. Thirty-nine birds were not vaccinated, and 43 birds were divided into 4 groups of 13, 10, 10 and 10 and received 0.5, 1.0, 1.5 and 2 ml vaccine respectively. On the 16th day following vaccination, the birds were again bled from the heart and the serum held in a frozen state until pre- and post-vaccination sera could be tested at the same time. The vaccinated and control birds were challenged by injecting 0.2 ml of virus intramuscularly. No evidence of infection occurred in the vaccinated group. Thirty-eight non-vaccinated controls died from the disease. *Standard hemagglutination inhibition* (HI) tests were performed on all paired sera. Incubation took place at room temperature and results of the tests were recorded at 15, 30 and 45 minutes. The hemagglutinating inhibition titers in 0.25 ml of undiluted serum were computed as the reciprocal of virus titer divided by the reciprocal of serum titer x dilution of the serum. *Serum neutralization* tests were performed in 8-10-day-old embryonating chicken eggs. Serial 10-fold dilutions of virus were prepared in nutrient broth. Equal parts of undiluted serum to be tested were mixed with each virus dilution. After standing 30 minutes, 0.1 ml of each virus-serum mixture was injected into the allantoic sac of 5 eggs. Viral infectivity was determined by mortality of the embryos. The *serum neutralizing titers* were the difference between the logarithms of titers of virus incubated with and without antiserum.

Results. The serological responses in chickens receiving the vaccine are shown in Table I. The HI titer rose gradually in the serum samples as the amount of vaccine given was increased. The average pre-vaccination HI titer was 1:14 which increased to average 1:635 sixteen days after vaccination. This represents a 45-fold increase in HI antibody titer.

The serum neutralizing antibodies against NDV also showed an increase in the post-vaccination serum samples. The average serum neutralizing index before vaccination was 1.22 in the 27 sera tested. The average index, taken on the 16th day after vaccination, was 4.07 or nearly a 4-fold increase over the average pre-vaccination index.

Discussion. Beta-propiolactone appears to be an ideal viricide with which to destroy the infectivity of Newcastle disease virus. Twenty-five thousandths per cent BPL was sufficient to destroy the infectivity of the virus and still leaving it capable of stimulating antibody to a high level to prevent disease. Lo Grippo and Hartman(2) have shown that the murine encephalomyelitis (MM virus), Eastern equine encephalomyelitis and rabies viruses were antigenic but no longer infectious to mice following treatment with 0.1% BPL. A greater concentration of BPL was required for inactivation of mouse brain suspensions of these viruses.

By using Newcastle disease virus and BPL the alteration of infectivity and antigenicity of the virus could be measured in the chicken, which is the natural host for the virus. The absence of infectivity with retention of high antigenicity indicated that BPL is ideal for manufacture of certain viral vaccines. This is especially true when viruses propagated in chicken eggs may become contaminated with other viral agents.

Summary. 1. A vaccine made by treating Newcastle disease virus wtih 0.025% beta-propiolactone was found not to be infectious to the chicken, its natural host. 2. The sera from chickens receiving the vaccine were found to contain protective antibodies 16 days following vaccination. 3. Paired sera collected before and after vaccination showed a marked rise in hemagglutination inhibition and neutralizing antibodies.

Journal Article 1844, Department of Microbiology and Public Health, Michigan Agri. Exp. Station

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Growth of *Micrococcus lysodeikticus* as Substrate for Lysozyme.*† (22241)

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Need for a procedure whereby large quantities of cells of *Micrococcus lysodeikticus* can be prepared conveniently for use as substrate for lysozyme, was encountered in studies of lytic enzymes from mammalian tissues(1). Present methods for production of *M. lysodeikticus* depend mainly on surface culture on agar media(2-6). Although a number of reports describe growth on liquid media(7-14), they are concerned primarily with nutritional requirements of the organism or pertain to mass cultivation without regard to lysability.

The present paper describes a simple procedure for production of cells of *M. lysodeikticus* in submerged culture. The influence of certain nutrients on growth and lysability of the cells is reported.

Methods and materials. M. lysodeikticus, strain No. 19 of the collection of the Institute of Microbiology, Rutgers University, was used throughout. All inoculations were made with 24 to 36 hour old agar cultures suspended in sterile, distilled water. Growth studies were carried out in test tubes (19 x 150 mm) containing 10 ml of medium. The tubes were set in a rack mounted on a rotary shaker and incubated at 28°C with agitation. Growth measurements were made with a Coleman Junior Spectrophotometer at 635 mμ. When quantities of cells were required, 250 ml Erlenmeyer flasks containing 100 ml of media were inoculated and incubated at 28°C on a rotary shaker. Method of preparation of dried cells and assay of lysozyme activity have been described previously(1). Crystalline egg white lysozyme was purchased from Worthington Biochemical Corp. The peptones used were: Lactalysate, Trypticase, Gelysate,

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* Paper of the Journal Series, N. J. Agri. Exp. Station, Rutgers University, State University of N. J., Departments of Agricultural Biochemistry and Agricultural Microbiology, New Brunswick.

† This work was presented in part before the Theobold Smith Society of the Soc. of Am. Bact. at Princeton University, Princeton, N. J., June 2, 1955.

Thiotone—Baltimore Biological Laboratories, Md.; N-Z Amine—Sheffield Farms, Inc., New York City; and Bacto-peptone, Bacto-tryptose, Neo-peptone, Proteose-peptone and Protone—Difco Laboratories, Detroit, Mich.

Results. M. lysodeikticus was cultivated on a medium containing yeast extract, 0.1%; glucose, 0.5%; NaCl 0.5% and Bacto-peptone, 0.5 or 1.0% at pH of 7.2 and on similar media with various components withdrawn singly to determine essentiality for growth. Development in the complete nutrient medium was satisfactory; however, by doubling the peptone content and omitting yeast extract and glucose, growth was even better. Omission of yeast extract from a medium with glucose resulted in definite inhibition of growth and may reflect a need for biotin(12). The most rapid development was obtained on a broth consisting of 1% peptone and 0.5% NaCl. Dried cell preparations from each medium were tested as substrates for lysozyme using 5, 10, 20 and 100 μg of crystalline enzyme in a 5.5 ml system at 25°C. Since linearity between enzyme concentration and activity begins to fall off above 20 μg of enzyme(1,15, 16), the activity on the linear part of the curve as well as non-linear portion may be compared. The most susceptible cells in respect to linear and extra-linear concentrations of enzyme were those from the medium which contained 1% Bacto-peptone and 0.5% NaCl. This medium also gave the best growth.

The results of tests when 10 different peptones were substituted singly for Bacto-peptone are shown by Fig. 1. No growth was obtained on Protone or Neo-peptone. Although there was greatest development with Bacto-tryptose, growth was preceded by a lag period of approximately 25 hours. Growth on Bacto-peptone showed the shortest lag. Cells from each of the 10 peptones were tested for susceptibility to lysozyme and rate of lysis at linear and non-linear concentrations of enzyme was greatest in cells which grew optimally. With cells from media supporting poor growth (curves 5 through 8), this relationship was not apparent. Susceptibility of cells from the medium containing Proteose-peptone was lower with high concentrations of the enzyme. This may reflect a decreased availability of sodium ion(16). Although rate and extent of growth on peptones 5 through 8 differed greatly, the cells were comparable with regard to lysability. Gelysate, for example, produced cells whose lysis was comparable to that of cells grown on Lactalysate which produced 2 times as much growth. Of the 10 peptones investigated, Bacto-tryptose[‡] and Bacto-peptone[‡] were chosen for further study.

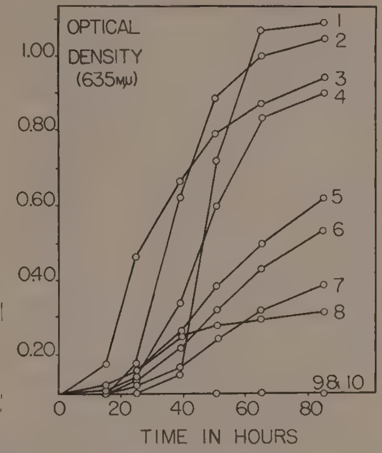

FIG. 1. Comparison of growth of *Micrococcus lysodeikticus* on 10 nitrogen sources. Curve 1—Bacto-tryptose, 2—Thiotone, 3—Bacto-peptone, 4—Proteose-peptone, 5—Lactalysate, 6—Trypticase, 7—N-Z Amine, 8—Gelysate, 9—Protone, 10—Neopeptone.

After demonstrating that the lag period for growth in Bacto-tryptose could not be eliminated by prior adaptation of the cells in this medium and that NaCl did affect the lag, cells were maintained on Bacto-tryptose agar and Bacto-peptone agar. These grew equally well when transferred to Bacto-peptone broth, however, addition of NaCl increased both extent and rate of development. Cells grown on Bacto-peptone agar and inoculated into Bacto-tryptose broth grew at a reduced rate compared to cells that had been maintained

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[‡] The composition of Bacto-peptone (Difco) and Bacto-tryptose (Difco) are presented in the Difco manual, Difco Laboratories, Inc. Detroit, Mich., 1953, pp. 256 and 262.

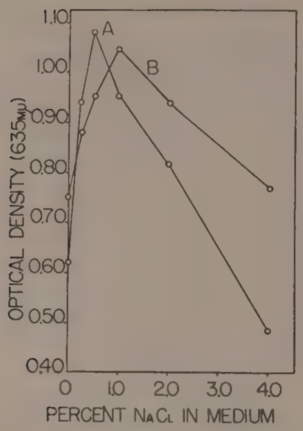

FIG. 2. Optimal concentration of NaCl for growth of *Micrococcus lysodeikticus* in Bacto-peptone and Bacto-tryptose media. Curve A, growth on Bacto-peptone; Curve B, growth on Bacto-tryptose.

on Bacto-tryptose agar. Cells maintained on Bacto-tryptose agar continued to show a prolonged lag period in Bacto-tryptose broth. This lag period was reduced markedly by addition of 0.5% NaCl to the medium. Cells that had been maintained on Bacto-peptone agar were influenced similarly by addition of NaCl.

The influence of concentrations of NaCl on growth of *M. lysodeikticus* in 64 hours is shown by Fig. 2. The optimal concentration of NaCl for growth was 0.5% with Bacto-peptone and 1% with Bacto-tryptose, even though Bacto-tryptose contains twice as much sodium ion and 10 times as much chloride ion as Bacto-peptone.‡

Tests of the activity of lysozyme on these cells indicated that susceptibility of cells grown on Bacto-peptone was greater than that of cells grown on Bacto-tryptose (Fig. 3). Bacto-peptone and Thiotone produced cells on which enzyme activity appeared to parallel growth. This was not the case for the other peptones.

Growth of *M. lysodeikticus* in Bacto-peptone broth was inhibited completely at pH 4 and 5. Although growth was most rapid at pH 7.5, it was comparable at pH 6 to 9 after 87 hours. Beers(14) also observed a high pH requirement for growth of this organism.

From the foregoing studies, the following medium is suggested for production of *M. lysodeikticus*: 1% Bacto-peptone and 0.5% NaCl in water at pH 7.5. Growth on this medium was compared with that on media recommended by Smolelis and Hartsell(6) and Saz and Krampitz(11). Agar was left out of the medium of Smolelis and Hartsell. All other components were equivalent. The inoculum was obtained from a Bacto-peptone-NaCl agar. The most rapid rate of growth was obtained on the recommended medium. There was considerable lag when *M. lysodeikticus* was grown in either of the other 2 media. The amount of growth was greatest on the medium of Smolelis and Hartsell. This is understandable since it contains as much as 6.5% available organic matter, as compared to 1.8% in the medium of Saz and Krampitz,

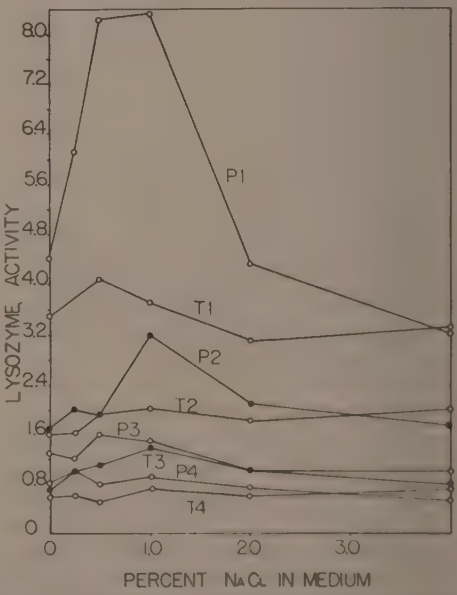

FIG. 3. Activity of crystalline lysozyme on cells grown on Bacto-peptone and Bacto-tryptose media containing various concentrations of NaCl.

Curve P1, cells from Bacto-peptone medium with 100 μg lysozyme.

T1	Bacto-tryptose	100
P2	Bacto-peptone	20
T2	Bacto-tryptose	20
P3	Bacto-peptone	10
T3	Bacto-tryptose	10
P4	Bacto-peptone	5
T4	Bacto-tryptose	5

TABLE I. Comparison of Lysozyme Activity on 3 Cell Preparations.

Medium used	Conc. of enzyme (μg)	Lysozyme activity* Δ % transmission from 30-60 sec
S and H†	5	.5
	10	.7
	20	1.5
	100	3.5
S and K‡	5	.7
	10	.7
	20	1.7
	100	5.0
Peptone-salt§	5	.8
	10	1.0
	20	2.0
	100	7.9

* Duplicate determinations.

† Smolelis and Hartsell medium without agar(6).

‡ Saz and Krampitz(11).

§ The recommended peptone-salt medium.

and only 1% in the recommended medium. It is likely that the lag in growth on the medium of Smolelis and Hartsell could be partially eliminated by adjustment of pH. Likewise, there might be more rapid development in the medium of Saz and Krampitz by addition of NaCl and adjustment of pH.

Table I shows that the lysability of cells from the recommended medium is greater than that of cells from the other 2 media.

Since Wilcox and Daniel(16) have indicated that a decrease from linearity in enzyme activity at high concentrations of lysozyme may be partially overcome by addition of sodium ions to either buffer or enzyme solutions, the influence of NaCl added to suspensions of cells which had been grown in media containing from 0 to 4% NaCl, was tested. The results are shown in Fig. 4. Doubling and quadrupling concentrations of NaCl in the suspension of cells grown in Bacto-peptone containing 0 to 1% NaCl caused a progressive inhibition of enzyme activity, but did not influence the lysability of cells which were grown in broth containing 2 to 4% NaCl. If, at a concentration of 100 μg lysozyme/0.5 ml, sodium ion is required to increase enzyme activity(16) then increasing the concentration of NaCl in the suspension should influence lysability of cells grown in the absence of NaCl. This was not the case. It is possible, therefore, that NaCl plays a structural role either in adsorption of the enzyme to the cell wall or in production of cellular substrate, or both; and consequently NaCl is more efficiently utilized if it is present during growth of the organisms.

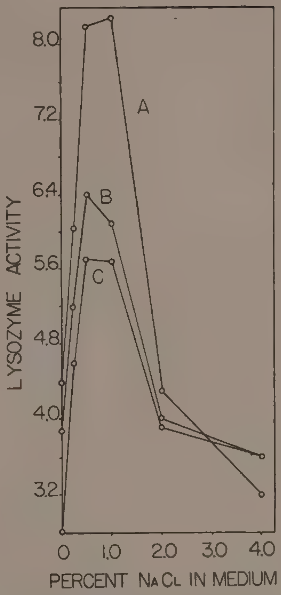

FIG. 4. Effect of adding NaCl to assay system on activity of crystalline lysozyme (100 μg). Cells grown in media containing varying amounts of NaCl. Curve A, .0171 M NaCl in assay system; Curve B, .0342 M; Curve C, .0684 M.

Summary. (1) A broth consisting of 1% Bacto-peptone and 0.5% NaCl at pH 7.5 is recommended for growth of *M. lysodeikticus* as substrate for lysozyme. (2) Cells grown and prepared by the recommended procedure were more susceptible to lysozyme than cells produced by other methods. (3) The rate of lysis of cells of *M. lysodeikticus* was affected by the concentration of NaCl used in the culture medium.

The technical assistance of Mr. Harvey Oshrin in the preparation of cells and media is gratefully acknowledged. The authors are indebted to Professor R. L. Starkey of the Department of Agricultural Microbiology for reading the manuscript.

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Effect of Ergotamine on Milk-Ejection in Lactating Rat.* (22242)

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It has been generally accepted(1-5) that the oxytocic principle from the posterior pituitary gland is the humoral agent responsible for "let-down" of milk from mammary gland. It has further been demonstrated(6) that adrenalin, fright, and other stimuli, interfere with this effect, whereas acetylcholine, when injected, acts similarly to "let-down" hormone(7-8). Evidence is presented in this paper that ergotamine also interferes with normal "let-down" of milk in lactating rats.

Materials and methods. Docile lactating rats, each with its first litter and weighing 230-260 g, were housed in individual cages and given Purina Dog Chow and water *ad libitum*. Seasonal greens were fed twice weekly. Each litter was reduced to 6 young shortly after birth and, whenever possible, these were equally distributed as to sex. Rats with less than 6 young were discarded. Ten control and 10 experimental animals each with litter of 6 were used. Ergotamine tartrate‡ was injected subcutaneously into lactating mothers 3 times a day at 6-hour intervals, from ninth through twelfth days postpartum, at dosage of 3 mg/kg/day. Half of the controls received comparable injections of physiological saline to test whether or not frequency of injection and/or amount of fluid injected would affect the pattern of maternal behavior. The remaining controls were uninjected. One and one-half hours following the second of 3 injections of the day, each litter was weighed, then isolated from the mother for 6 hours during each day of injection period. The young were kept warm to prevent any undesirable effects from exposure. In some preliminary experiments, stomachs of several litters, killed at end of 6-hour isolation period, contained no milk. One and one-half hours after third injection of the day, each litter was again weighed to determine weight loss during the 6-hour interval. The young were then placed back with the mother and allowed to suckle for exactly 20 minutes. Length of time before each mother began nursing was recorded. The litter was then removed and weighed to determine amount of milk obtained during the 20-minute interval. (Table

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* Part of dissertation submitted to Graduate School of Arts and Sciences of University of Cincinnati in partial fulfillment for degree of Doctor of Philosophy.

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‡ Kindly supplied by Sandoz Chemicals, N. Y.

TABLE I. Average Quantity of Milk, Expressed as % Body Weight Gain, Secured in 20 Min. by Litters of 10 Control Lactating Rats, and 10 Experimental Animals Injected with Ergotamine from the 9th to 12th Days Postpartum.

Day of lactation	Group	%	S.E.	P
9	Control	5.59	.87	
	Treated	.36	.53	.001
10	Control	4.03	.54	
	Treated	2.22	.84	.1
11	Control	4.18	.69	
	Treated	2.46	.90	.2
12	Control	2.87	.82	
	Treated	2.27	.79	.7
Mean	Control	4.17	.73	
	Treated	1.83	.76	.001

I). All young were killed on twelfth day postpartum immediately after they had suckled. Their stomachs were opened and semisolid curds removed and weighed as a check against amount of milk calculated by weighing litters before and after sucking.

Results. Control offspring lost an average of 2% (±.15%) of their body weight during isolation period of 6 hours on each of days 9 through 12 postpartum, whereas litters of experimental group lost 2.2% (±.14%) during the same period. This difference is significant ($P = .02$). There is, however, no apparent difference between the 2 groups in the time it takes for mothers to commence nursing after their offspring have been returned to them following the isolation period of 6 hours. All mothers were nursing their young within 5 minutes. The quantity of milk obtained by experimental litters on the first day of treatment was very small in comparison with that obtained by control offspring on the same day. The difference was highly significant ($P = .001$). This difference progressively diminished, however, following additional treatment on days 10, 11, and 12 as animals became more resistant to the drug. ($P = .1$, .2, and .7 respectively.) The average amount of milk obtained over the 4-day period of treatment by experimental rats, nevertheless, was significantly less ($P = .001$) than that obtained by control offspring over the same period.

The combined weights of the semisolid curds, removed from the stomachs of each litter after they had been killed averaged 20% less than values calculated from weighing litters before and after the 20-minute period of nursing (Table II). This indicated that the method used in determining quantity of milk secured by young was accurate.

Discussion. It was expected that young of ergotamine-treated rats would lose a significantly higher percentage of body weight than control animals during the 6-hour isolation period. Offspring of treated rats are significantly lighter in weight at end of period of treatment than are control litters. Being of smaller size, there is a relatively greater surface area for heat loss than in larger control offspring. Greater energy, therefore, is expended by the smaller animal to maintain integrity of its internal environment.

It is well known(9-12) that with interruption of suckling, the mammary gland becomes engorged with milk. It was observed in the present study that when young were isolated from the mother for 6 hours and then replaced, the mother hastily gathered her young for feeding, apparently to relieve the turgidity in her mammary glands.

It was observed that within 20 minutes after being so isolated, young of untreated animals stopped sucking. Offspring of ergotamine-treated rats, however, were still sucking actively at the end of this period and had to be pulled away from nipples. It was apparent that they had not secured all the milk they wanted. The results for untreated litters (Table I), though not obtained under identical conditions, are similar to those obtained by other investigators(13-14).

Our studies(15) have shown that ergotamine does not impair synthesis of milk in mammary gland nor does it affect behavior of

TABLE II. Comparison of Calculated Quantity of Milk Obtained on 12th Day Postpartum and That Obtained from Stomachs of Litters of 10 Control Lactating Rats, and 10 Experimental Animals. Data expressed as % body wt gain.

Group	Calculated	Obtained	% difference
Control	2.87	2.24	21.9
Treated	2.27	1.84	18.9

the mother as evidenced by incidence of sucking during period of treatment.

Ergotamine apparently interferes, in some way which can only be explained theoretically, with release of milk from the gland. It has been demonstrated that definite contractile myoepithelial cells are present in mammary glands of the rat which contract apparently in response to oxytocin released from the posterior pituitary gland by nerve impulses stimulated by nursing(16-18). These cells are abundantly distributed around the alveoli and ducts and are not innervated. They are, furthermore, ideally arranged for squeezing milk out of acini and from small ducts into large ducts.

It is therefore likely that ergotamine may exert its inhibitory influence on milk "let-down" in rat mammary gland by interfering in some way with normal action of oxytocin on myoepithelial cells. In view of our results, further experimentation is planned with hope that the mechanism of ergotamine inhibition of milk "let-down" will be clarified.

Summary. 1. The quantity of milk secured in 20 minutes by litters of ergotamine-treated rats on the first of 4 days treatment was small in comparison to that obtained by control offspring on the same day. The difference was significant. This difference progressively diminished, however, following additional treatment on the following 3 days, as the animals became more resistant to the drug. The average amount of milk obtained by experimental animals over the 4-day period of treatment, nevertheless, was significantly less than that obtained over the same period by control offspring. 2. The possible mechanism by which ergotamine interferes with "let-down" of milk from rat mammary gland is discussed.

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Regional Differences in Propagation of Spreading Cortical Depression in the Rabbit.* (22243)

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Leão(1) reported that in the rabbit spreading depression (SD) invaded the entire exposed surface of the stimulated hemisphere with the single exception of a small region medial to the parasagittal sulcus. This region appeared to be coextensive with area Rsg β of M. Rose(2), also with area 29d of Brodman(3) and area u of Droogleever-Fortuyn (4). Evidence has been presented by J. E. Rose and Woolsey(5) that this region is part of the rabbit's cingular area, the rest of which is situated on the medial aspect of the hemisphere.

In the present paper that part of the cingular area which is situated on the dorsal surface of the hemisphere will be indicated by "Cg." The dorso-lateral aspect routinely exposed, exclusive of Cg, will be called the "convexity" of the hemisphere. The invasion of Cg by SD was investigated, using as a criterion the slow potential change (SPC) which accompanies this phenomenon, in addition to the changes in the electrocorticogram (ECG) used by Leão. Marshall and his coworkers have shown, mostly in cats and monkeys in which SD is less easily obtained than in rabbits(6,7), that the production of this phenomenon can be facilitated in several ways (8-13). After having confirmed Leão's finding that SD does not normally propagate into Cg, it was attempted to make this area exhibit SD by application of the procedures described by Marshall *et al.*

Method. Rabbits were used exclusively. Cannulation of the trachea, jugular vein and femoral artery, and exposure of the cortex were performed under ether narcosis. During recording, the preparation was immobilized with Squibb's Intocostrin (about 5 units/kg bodyweight/hour) and maintained on artificial respiration. Blood pressure was recorded from the femoral artery when necessary. The electrodes were placed as shown in Fig. 1A. Electrode 1 was used for stimulation. Bipolar ECGs were led off from the convexity of the hemisphere and from Cg with electrode pairs 2-3 and 4-5, respectively. Slow potential changes with respect to an indifferent electrode contacting the pinna were recorded from electrodes 3 and 5. Recording and stimulating electrodes were of the silver-silver chloride type mounted on springs and resting lightly on the cortex. Electrocorticograms were recorded with an Offner electroencephalograph, slow potential changes were fed into d.c. amplifiers(14) and recorded on photographic paper with d'Arsonval-type galvanometers. Galvanic stimuli (3 to 5 volts), lasting for 2-3 seconds, were used.

Results. Spreading Depression in the Dorsal Part of the Cingular Area (Cg). The first stimulations elicit on the convexity of the

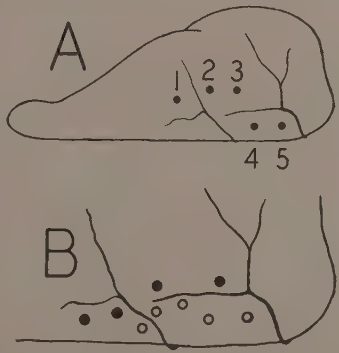

FIG. 1. A, a dorsal view of rabbit's hemisphere. Electrode placements are indicated by dots. Electrode 1 was used for stimulation. From electrodes 2 and 3 (on "convexity" of the hemisphere) and from electrodes 4 and 5 (on cingular area) bipolar electrocorticograms were led off. Slow potential changes were recorded from electrodes 3 and 5. B shows on a larger scale the cingular area on dorsal aspect of hemisphere. Electrode placements indicated by dots yielded typical slow potential changes, those indicated by open circles did not.

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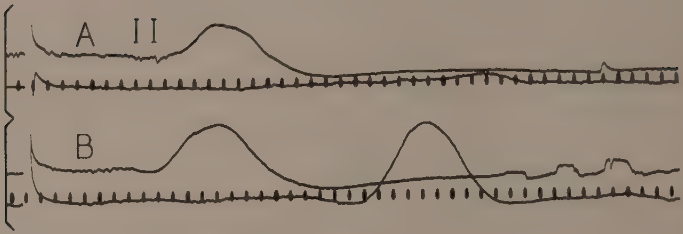

FIG. 2. Two slow potential change records, A and B. In each the upper trace is from convexity of hemispheres, lower trace from cingular area. A was made 33 min. and B 43 min. after start of infusion of 90% sucrose solution. Time marks indicate 10 sec intervals. The first 5 mV calibration line is for the upper, the second for the lower trace of each record.

hemisphere marked and often complete depressions of the spontaneous ECG accompanied by well developed slow potential changes (SPC). As the number of stimulations increases, depression tends to be replaced more and more by convulsoid patterns (1,6,15). The invasion of Cg by spreading depression (SD) was examined in 36 preparations. In 35 of them no typical SD or SPC invaded Cg. In most cases there was not the slightest indication of a slow potential change. In 9 preparations, however, a slight deflection was present in the SPC record from Cg. This small deflection appeared 1½ to 3 minutes later than the SPC recorded from the convexity of the hemisphere with electrode 3. Fig. 2A shows the relation in magnitude of the SPC recorded from the convexity and the small deflection recorded from Cg. A few spikes were sometimes observed in the ECG from Cg when the depression on the convexity of the hemisphere had been replaced by convulsoid activity. This spike activity, when present, appeared considerably later in Cg than on the convexity. Only in one preparation there was recorded from Cg a SPC comparable in size with that obtained from the convexity of the hemisphere. It was accompanied by a marked depression of the ECG. An attempt to delineate the region which does not produce the usual symptoms of SD is hampered by the long intervals between stimulations which are necessitated by the long refractory period of SD. Ten minute intervals were used in this investigation. Fig. 1B shows the results of an attempt to outline this region. It would appear that the limits of the unresponsive region correspond with that part of the area cingularis of J. E. Rose and Woolsey which is situated on the dorsal aspect of the hemisphere. In a few experiments the frontal boundary of the unresponsive area was traced as far as feasible on the medial aspect of the hemisphere. This boundary also appeared to coincide with that of Cg.

Effects of exposure of the cortex. In 7 preparations records made 2 to 3 hours after exposure of the cortex were not markedly different from the traces obtained shortly after removal of the dura. They showed clearcut SD and SPC in the traces recorded from the cortical convexity, no typical SD or SPC in Cg. Since long exposure of the cortex facilitates SD in monkeys(11) and cats(6,7) it was attempted to elicit this phenomenon in Cg of the rabbit in the same way. Two experiments were performed. In one, typical SDs and SPCs developed in Cg 8½ hours after exposure. They were present for two hours, after which the experiment was terminated. The other preparation examined for a similar period of time did not show a clearcut SPC in Cg.

Effects of intravenous administration of concentrated sugar solutions. Marshall(9) reported that dehydration of the cortex by slow infusion of a 90% sucrose solution facilitated the production of SD in cats. In the present experiments sucrose solution (90%) was infused through an inlying cannula into the jugular vein at a rate of 1-2 cc/min., causing a moderate blood pressure rise, profuse diuresis and in one preparation marked ascites. The brain shrank visibly. Three preparations were used, none of which exhib-

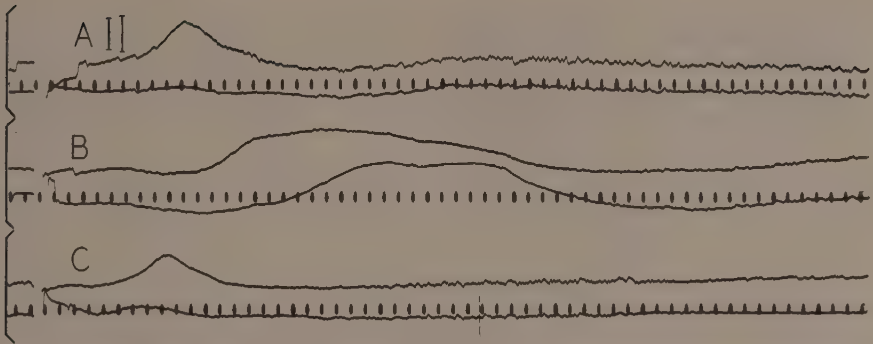

FIG. 3. Three slow potential change records, A, B and C. In each the upper trace is from convexity of hemisphere, the lower from cingular area. Record A was taken while cortex was covered with mineral oil near body temperature, B while cooled oil passed over cortex, and C again while covered by oil near body temperature. Time marks indicate 10 sec intervals. First 5 mV calibration line is for the upper, second for the lower trace of each record.

ited a typical SPC or SD in Cg before infusion. However, after 43, 50 and 53 min. of infusion a large SPC was recorded from Cg, accompanied by an incomplete but definite depression of the ECG. Fig. 2, A and B are SPC records produced by stimulations 33 and 43 minutes after the start of sugar infusion in one of these experiments. The upper trace of record A shows the well developed SPC led off from the convexity of the hemisphere. The lower trace recorded from Cg shows the small deflection mentioned above. Record B, taken 10 minutes later shows large SPCs of both the convexity and Cg, separated by a time interval of about 2 minutes. The duration of the depressions of the ECG recorded from the convexity of the hemisphere increased considerably during dehydration. Also, the height and duration of the SPC led off from the convexity were increased.

Effects of cooling of the cortex. Cooling the cortex was found by Marshall, Essig and Dubroff(10), to facilitate the production of SD. In the present experiments a skin well was formed over the exposed cortex by sewing the edges of the skin wound to a steel ring suspended a few cm. above the skull. The well was filled with oil near body temperature. Then cold oil (about 10°C) was passed through the well until a steady temperature of the outflowing oil was reached (17-20°C). Next the well was filled with warm oil again. After each change in oil temperature the cortex was stimulated. Fig. 3 is taken from such an experiment. Record A shows SPC traces from the convexity of the cortex (upper trace), and Cg (lower trace) respectively, while oil near body temperature filled the well. Record B was taken when the outflowing oil had reached a temperature of 18°C; and record C was made after a subsequent change to warm oil. Under oil near body temperature there is no obvious deflection in the SPC record from Cg, but after cooling there develops a large SPC of long duration which starts about 1 minute after the SPC recorded from the convexity. The SPC recorded from the convexity during cooling shows an increase in height and especially in duration (Fig. 3B, upper trace), as compared with the SPC led off from the same location under warm oil. Cooling increased the duration of the depression of the ECG from the convexity as much as 2 to 3 times, and had a tendency to decrease the convulsoid activity when present.

Effects of treating the cortex with isotonic sucrose solution. Essig and Marshall(8) reported that in cats and monkeys the production of SD is facilitated by treatment of the cortex with an isotonic sucrose solution. In the present experiments a solution of 10% sucrose in distilled water was made to flow at a rate of 10 to 12 cc/min. through the skin well described above. Since the solution has a high specific resistance, recording of ECG and SPC can be continued during this procedure. Six experiments were successfully completed. Before application of the sugar solution typical spreading depressions were recorded from the convexity of the hemisphere but in none of the preparations from Cg. In all instances a sizeable SPC and definite depression of the electrocorticogram were recorded from this area after the cortex had been covered for some time (10 to 20 min.) by the sugar solution. The first stimulation after application of the sugar solution produced in each case a depression of the ECG of unusually long duration. Subsequent SD were of shorter duration and after 40 to 50 minutes of continued application of the sugar solution the depression was approximately of pre-treatment length. The SPC recorded from the convexity tended to be somewhat larger and of longer duration during the sugar application. In three experiments the sugar solution was replaced by Ringer's solution for about 10 minutes. The subsequent cortical stimulation caused a SD which was not propagated into Cg.

Effect of Ringer's Solution with 10 times normal potassium concentration. Treatment of the cortex with Ringer's solution containing more than the normal amount of potassium was also found to facilitate SD by Marshall *et al.*(12,13). In the experiments to be reported, KCl was added to normal Ringer's solution to make the K concentration 10 times normal. The solution was made to flow through the skin well at a rate of 10 to 12 cc/min. This treatment of the cortex caused spontaneous convulsive activity which, as was observed previously(16,17) can, and did act as stimulus for "spontaneous" SDs. By limiting the time of exposure in 2 experiments to 10 and 8 minutes, it was possible to avoid the convulsive activity so far that regular spreading depressions could be elicited. These preparations, which before the application of the K solution did not show a typical SPC or SD in Cg, did so after the cortex had been treated. After K treatment the magnitude of the SPC and the length of SD recorded from the convexity of the hemisphere were not increased. The cortex of the preparation with the 8 min. exposure was stimulated at 10 min. intervals following removal of the K solution. Only the first 2 stimuli produced typical SPCs in Cg; later stimuli although eliciting large SPCs on the convexity of the hemisphere failed to produce SPCs in Cg. The facilitation of the SD caused by exposure to the K solution thus seemed to be spontaneously reversible.

TABLE I. Latent Periods of Asphyxial Potentials from Convexity of Hemisphere and from Cingular Area (Cg).

No.	Convexity	Cg	Difference
1	310	460	150
2	125	230	105
3	130	270	140
4	140	280	140
5	120	160	40
6	80	85	5

Length of time, in sec between onset of asphyxia and development of the asphyxial potential. In preparations 1 to 5 the cingular area did not exhibit spreading depression; in preparation 6 it did.

Asphyxial potentials from the dorsal part of the cingular area. From 2 to 5 minutes after cortical asphyxiation a potential variation can be recorded from the cortex of similar magnitude and general appearance as the SPC accompanying the SD. Evidence has been advanced for the thesis that this asphyxial potential is related to the SPC accompanying SD(18-20). Since Cg is not normally invaded by the SD it was of interest to compare the asphyxial potential from the cortex on the convexity with the effects of asphyxiation of Cg. Six experiments were successfully completed. In 5 of them the SD did not invade Cg; the 6th experiment was the one, mentioned above, in which SPCs and depressions were recorded from this area. In all experiments an asphyxial potential was elicited both from the convexity and from Cg. However, in the 5 cases in which the SD had not invaded Cg the asphyxial potential developed from 40 to 150 sec. later in this area (Table I). In the experiment in which Cg exhibited SD the asphyxial potentials developed almost simultaneously.

Discussion. It is of interest that the limits of the area which is not normally invaded by SD correspond as far as investigated with the boundaries of the cingular area(5). There thus seems to exist a relation between the presence or absence of SD and the anatomy of the cortex as given by cytoarchitectonic criteria.

On occasion a small deflection in the SPC record from Cg was observed. Since Cg is a strip of cortex only a few mm wide, electrodes placed on it are in general quite close to its lateral boundary in the parasagittal sulcus. Immediately lateral to the sulcus typical SDs and SPCs can be recorded. The distances involved are so small that the possibility has to be considered that the electrodes on Cg are affected by the physical spread of electrical phenomena occurring in the cortex immediately lateral to the sulcus. The time interval between the typical SD and SPC recorded from the hemispheral convexity and the atypical phenomena from Cg is commensurate with the time of propagation of the SD from the electrodes on the convexity to the cortex immediately lateral to the parasagittal sulcus. This explanation is consistent with experiments in which the propagation of SD was halted by a cortical cut(21); a small deflection similar to the one observed in the above experiments was recorded immediately across the cut on the non-stimulated side.

When after repeated stimulation the depression on the convexity of the hemisphere had been replaced by a convulsoid pattern, spike activity was sometimes observed in Cg. It seems possible that this spike activity was propagated actively from the cortex lateral of the parasagittal sulcus into Cg, since it is known that such convulsoid patterns are propagated to cortical areas not primarily affected(22). On the other hand, spread of a typical SPC into Cg is visualized as a change of this area, which makes it possible to be invaded by SD.

The 5 procedures which tend to facilitate the propagation of the SD in Cg produced changes in SD and SPC recorded from the convexity of the hemisphere which varied a great deal. Intravenous sucrose (90%) administration produced a gradual increase in the duration of the depression of cortical activity long before SD was propagated into Cg. Cooling caused a 100 to 200% lengthening of the depression. Treatment with isotonic sucrose solution produced an immediate prolongation of the depression, decreasing with the duration of the sugar application. The Ringer's solution with increased potassium concentration was without apparent effect on the length of the depression. Treatment of the cortex with isotonic sucrose solution, the intravenous administration of 90% sucrose, and especially cooling, increased and prolonged the SPC. The 5 procedures which facilitate the propagation of the SPC in Cg may not do so in the same manner. In the propagation of SD there may be involved a chain of events which can be facilitated at several points. For instance, it is conceivable that dehydration by bringing individual cortical elements closer together enhances the effect of one element on another. Or, if depolarization of neuronal elements is a necessary step in the propagation of SD(21) then this may be facilitated by the treatment with greater than normal potassium concentrations. If it be assumed that the 5 procedures facilitate via the same mechanism, then it follows from the varied effects of these procedures on the SPC of the convexity that this mechanism is not that underlying the changes in size, shape or duration of the SPC.

It was observed that the asphyxial potential develops later in Cg than in the cortex on the convexity of the hemisphere (in those preparations in which Cg is not invaded by SD). Assuming that the SPC accompanying the SD and the asphyxial potential are based on the same mechanism (18-20), then both this time difference and the fact that SD does not normally invade Cg may be indicative of the greater difficulty with which this mechanism is set in motion in Cg as compared with the cortex on the convexity.

Summary. 1. Of 36 rabbits consistently exhibiting spreading depressions and slow potential changes in response to galvanic stimulation of the cortex, only one exhibited propagation of these phenomena into that part of the area cingularis which is situated on the dorsal aspect of the hemisphere. Occasional small deflections recorded from this area were interpreted as evidence of physical

spread of typical slow potential changes in nearby cortex. 2. Where previously absent, typical slow potential changes and spreading depressions were produced in the cingular area by a) prolonged exposure of the cortex, b) intravenous infusion of concentrated sucrose solution, c) cooling of the cortex, d) treatment of the cortex with isotonic sucrose solution, and e) with Ringer's solution containing 10 times the normal amount of potassium. 3. Asphyxial potentials were recorded both from the cingular area and from the cortex on the convexity of the hemisphere. However, the latent period of this potential from the cingular area was markedly longer than that recorded from the convexity.

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Enzyme Studies in Muscular Dystrophy. II. Muscle Dipeptidase Activity and Vitamin E-Deficiency.* (22244)

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Vit. E-deficiency in the rabbit and other species results in characteristic muscular wasting and weakness. In previous investigations, on a number of tissues of the deficient rabbit, we found the cathepsin activity, *in vitro*, to be markedly increased in muscle, but unchanged in the other tissues(1). To investigate further the metabolism of protein in this form of muscular dystrophy, we have studied the activity of two previously characterized peptidase systems of muscle, glycyl-l-leucine and glycylglycine dipeptidase(2-4).

Methods. Vit. E-deficiency was induced in young rabbits by maintaining them on Diet 11 of Goettsch and Pappenheimer(5) treated with 1% ethereal $FeCl_3$. Control animals received the untreated diet supplemented with 25 mg % dl-α-tocopherol. After the appearance of symptoms of Stage II(6) of muscular

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TABLE I. Effect of Vit. E-Deficiency on Dipeptidase Activity of Muscle Extracts.

Substrate	Extract	Buffer	0.001M activator	Control No. animals	Control No. determ.	Control Specific activity†	Vit. E-deficient No. animals	Vit. E-deficient No. determ.	Vit. E-deficient Specific activity	t	P*
						60 min.			60 min.		
Glycyl-l-leucine	KCl	$PO_4^{\equiv}$	—	7	14	100 ± 38‡	6	10	410 ± 235	5.2	<.01
			+Mn^{++}	4	8	85 ± 29	5	8	300 ± 213	3.0	”
	H_2O	”	—	3	12	155 ± 100	7	17	540 ± 250	5.3	”
			+Mn^{++}	3	6	75 ± 32	4	8	390 ± 237	5.3	”
						120 min.			120 min.		
Glycylglycine	KCl	”	—	5	10	65 ± 36	7	14	100 ± 59	1.8	<.1
			+Co^{++}	5	10	85 ± 32	7	14	410 ± 243	4.5	<.01
		Veronal	—	4	8	60 ± 10	7	14	80 ± 48	1.1	<.3
			+Co^{++}	4	8	80 ± 26	7	14	205 ± 99	3.8	<.01
	H_2O	$PO_4^{\equiv}$	—	4	8	110 ± 46	6	12	94 ± 37	1.0	<.4
			+Co^{++}	4	8	125 ± 53	6	12	405 ± 200	4.2	<.01
						mg protein N/ml			mg protein N/ml		
Protein nitrogen content of extracts	KCl			12		3.05 (2.19-3.72)§	12		2.45 (1.68-3.33)		
	H_2O			15		1.84 (1.41-2.52)	18		1.86 (.89-2.69)		

* P = Level of significance. † Specific activity = % hydrolysis/mg of muscle extract protein nitrogen/ml of incubation mixture. ‡ Stand. dev. § Figures in parentheses are range of values.

TABLE II. Effect of Vit. E-Deficiency on Dipeptidase Activity of Muscle Homogenates.*

Substrate	.001M activator	Control No. of animals	Control mg PN,† g wet wt	Control Activity,‡ g wet wt	Control Specific activity	Vit. E-deficient No. of animals	Vit. E-deficient mg PN, g wet wt	Vit. E-deficient Activity, g wet wt	Vit. E-deficient Specific activity
			a	b	b/a		a	b	b/a
Glycyl-l-leucine	—	4	23.5 (21.3–25.2)§	810 (710–1080)	34 (29–44)	3	19.2 (16.4 23.0)	5910 (2200–10800)	310 (95–660)
	Mn^{++}	3	24.2 (23.6–25.2)	700 (495–840)	29 (20–36)	3	19.2 (16.4–23.0)	3410 (1470–5950)	175 (65–365)
Glycylglycine	—	4	23.5 (21.3–25.2)	160 (0–480)	7 (0–20)	4	20.7 (16.4–25.0)	184 (90–280)	9 (5–16)
	+Co^{++}	4	23.5 (21.3–25.2)	350 (200–500)	15 (8–21)	4	20.7 (16.4–25.0)	2350 (500–4000)	110 (22–250)

* All muscle homogenates were prepared in H_2O. † PN = Protein nitrogen. ‡ Activity = % hydrolysis of glycyl-l-leucine at end of 60 min. incubation period and of glycylglycine at end of 120 min. of incubation. § Figures in parentheses are range of values.

dystrophy, the animals were sacrificed. Mixed portions of the muscles of the leg, thigh and back were homogenized with 2 volumes of either water or 2% KCl. The enzyme source was either a muscle extract obtained by centrifuging at 1400 x g, or the whole muscle homogenate. The extracts were diluted to 5-15 times their original volumes, and activity was determined at 2 or more different dilutions. The homogenates were diluted to 5-10 times their original volumes and strained through gauze. The observed values of activities of duplicate aliquots were averaged. Protein nitrogen content of all of the preparations was determined by a colorimetric nesslerization procedure. The assay system consisted of 0.15 ml buffer (1 M phosphate, pH 7.4-7.6 or 0.015 M veronal, pH 7.7-7.9), 0.15 ml 0.01 M metal ion activator when used, 0.5 ml 0.1 M substrate, 0.5 ml of the enzyme source, and sufficient water to make the final volume 1.5 ml. The mixtures were incubated at 37.5 ± .5°C for periods of one and 2 hours. Hydrolysis of the dipeptides was determined by the colorimetric ninhydrin procedure of Moore and Stein(7) as adapted by Schwartz and Engel(8). Preparations containing the enzyme source showed no measurable change in ninhydrin reacting material in the absence of substrate.

Results. Dipeptidase activities of the muscle extracts are shown in Table I and those of the whole homogenates are given in Table II. The incubation periods used were relatively short as compared to previous studies on these systems. Although the rates of hydrolysis of both substrates decreased somewhat during the second hour of incubation, as previously reported for rat muscle(9), the hydrolysis of glycylglycine had to be determined over a 2-hour period in order to obtain measurable values in the control group. Hydrolysis of glycyl-l-leucine is reported for one hour periods of incubation although in many experiments activity during the second hour also was determined. The addition of Mn^{++} did not increase glycyl-l-leucine hydrolysis by either the normal or E-deficient tissues, and, in fact, was somewhat inhibitory. We do not know why the Mn^{++} activation of glycyl-l-leucine dipeptidase activity reported by Smith(2,4) was not observed in these experiments; however, it is noteworthy that in at least one instance, this investigator also failed to note any stimulation by Mn^{++}(2). The addition of Co^{++} caused a slight increase in glycylglycine hydrolysis by the control group and a considerable increase by the E-deficient group. During vit. E-deficiency there was a marked increase in dipeptidase activity of the rabbit muscle extracts and whole homogenates. The increase in glycyl-l-leucine dipeptidase activity was not dependent upon the addition of Mn^{++}, but the increase in glycylglycine dipeptidase activity during vit. E-deficiency required the addition of Co^{++} activator. Elevation in dipeptidase activity was observed during both the first and the second hours of incubation, although the absolute rates of hydrolysis by both the control and deficient groups decreased slightly during the second hour. In one experiment, hydrolysis of glycyl-l-leucine by a mixture of extracts from control and vit. E-deficient rabbit muscle was equal to the sum of hydrolysis by the individual extracts. This result suggests that the difference between the activities of normal and E-deficient muscle was not due to the presence of either an inhibitor in the control group or of an activator in the deficient preparations. Although there was usually some decrease in protein nitrogen content of the muscle during the deficiency, which could give slightly higher values for the specific activity, this relatively small difference in nitrogen content cannot account for the large increases in dipeptidase activity we have observed.

An increased incorporation of glycine into muscle protein, *in vivo*, occurs, at least over short periods of time, in deficiency of vit. E (10). Therefore, the decrease in muscle mass that is observed in this condition appears to be due to an even greater increase of protein catabolism. Both dipeptidase and catheptic (1) activity of muscle is increased *in vitro* during the deficiency. It has been demonstrated that purified cathepsins have the ability, under proper conditions, to synthesize polypeptides *in vitro* by transpeptidization (11,12). While the functioning of these enzymes *in vivo* is yet to be demonstrated and other and more important enzyme systems are probably involved in protein metabolism, it is plausible to speculate that the net breakdown of muscle protein, observed *in vivo* during vit. E-deficiency, may be due to increase in rate, and a shift in the equilibrium of a reversible enzyme system(s) catalyzing peptide bond formation and breakdown.

Summary. The effect of vit. E-deficiency on hydrolysis of glycylglycine and glycyl-l-leucine by skeletal muscle extracts and homogenates was investigated. During this deficiency there was a marked increase in dipeptidase activity of both muscle extracts and homogenates. The increase in glycylglycine dipeptidase activity could be demonstrated only after the addition of Co^{++}.

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Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). (22245)

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In tissue culture studies, the determination of the amount of growth under varying environmental conditions is often of central importance. In this laboratory, the actual enumeration of the cells(1) proved a useful but laborious procedure(2-4); and studies were therefore undertaken to devise a rapid yet reliable method of measuring growth. Consideration was given to the direct turbidimetric measurement of the resuspended cells, the determination of total nitrogen by nesslerization, or of cell protein by precipitation procedures; but the method to be here described proved superior to any of these in its simplicity and reproducibility. Essentially, the technic is a modification of the colorimetric method of Lowry, *et al.*(5) for measuring protein, using a phenol reagent (Folin-Ciocalteau) for the development of color. As here described, the method is designed for use in cultures adherent to a glass surface, and overlaid with a fluid medium.

Materials. 1. Earle's salt solution(6) (or any similar balanced salt solution) for washing the cell layer in the culture flask. 2. Alkaline copper solution to dissolve the cells. This is kept as 2 stock solutions, A and B: A, Na_2CO_3, 200 g; NaOH, 40 g; NaK tartrate, 2 g; q.s. to 10 liters; B, $CuSO_4 \cdot 5H_2O$, 5 g; q.s. to 1 liter. (The tartrate was incorporated with the alkali, rather than the $CuSO_4$, because of the slow development of a precipitate in the copper-tartrate mixture.) Solutions A and B are mixed in the proportion of 50 parts of A to 1 part of B to form Lowry's solution C, which is prepared fresh daily.

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3. Folin-Ciocalteau reagent† (7). The reagent should be standardized by determining that concentration which gives the maximum and most stable color. This standardization need be carried out only once for each lot. Crystalline bovine albumin may be used as the reference standard. One part of a solution at 50 μg/ml is added to 5 parts of solution C. Six ml are then distributed into each of 6 cuvettes, and a parallel series is prepared using H_2O instead of the protein solution. Dilutions of the F-C reagent are prepared as indicated below:

Reagent	5	5	5	5	5	5
H_2O	4	5	6	7	8	9
Dilution	5/9	5/10	5/11	5/12	5/13	5/14

0.5 ml of each of these dilutions is rapidly blown into 1 cuvette† containing the protein solution, and 1 control cuvette containing H_2O. The color is read after 15, 30, 60, 90, 120, 180, and 240 minutes, in each case against the corresponding water control. That dilution of the F-C reagent which reaches maximum color within approximately ½ hour, and remains stable for 2 hours thereafter, is the one used in the test.

Details of procedure. Step 1. Washing of cultures. The culture flasks are drained of medium by inversion, and the adherent cell layer washed twice with Earle's salt solution (6). For the culture flasks previously described (2,8), with a surface area of approximately 15 sq cm, and a 15 ml capacity, an 8 ml wash is used. After the second wash the flasks are left inverted over clean gauze to drain for approximately 15 minutes, when clean stoppers are inserted. At this stage, the washed and dry cells may be stored in the original culture flasks for several days with no change in the protein analysis. *Step 2. Solution of cells in alkaline copper tartrate.* Ten ml of Lowry's reagent C (5) are added to the flasks with an automatic syringe and allowed to remain in contact with the cell layer for 10 minutes, after which the flasks are shaken manually to complete the dissolution of the cells. At this stage also, the flasks may be stored for several days with no loss in protein. *Step 3. Color development.* A biuret color is produced by the interaction of the alkaline copper sulfate tartrate solution C with the protein; and the intensity of this color may serve as a rough measure of the amount of cells, and thus of the amount of the solution which must be used in the final test in order to produce a color within the optimum measurable range (optical density $= <0.4$). When the color is not detectable, 2 ml is taken for analysis; when the color is pale violet, 0.5 ml will suffice; while with a deep violet color, the solution should be diluted with 1 or 2 volumes of solution C, and 0.5 ml taken for analysis. The appropriate amount of the cell solution is pipetted into a Coleman cuvette,† and solution C added to a total of 5 ml. One ml of H_2O is then added, and finally, 0.5 ml of the properly diluted F-C reagent is jetted in by a syringe. The rapid admixture of the color reagent with the solution is essential; and the slow addition of the reagent without such rapid admixture leads to erroneous results. The controls consist of 3 cuvettes, each containing 1 ml of crystalline bovine albumin at 50 μg/ml instead of the H_2O used in the test proper. A water blank is also run. Absorption is read after 30 minutes at 660 mμ; but the color remains stable for 2 hours.

Calculation of results. Results of the test are most simply expressed in terms of the bovine albumin equivalent of the unknown cell culture. For 4 different cell lines the ab-

TABLE I. Conversion of "Bovine Albumin Equivalent" of 8 Tissue Culture Strains to Cell Mass, Nitrogen and Number.

Cell line	Multiplying factors to convert bovine albumin equivalent (mg) to Cell mass (mg)	Cell N (mg)	Cell count ($\times 10^6$)
Mouse fibroblast (Earle)	1.4	.18	.12
HeLa (Gey)	1.4	.16	.16
Intestinal epithelium (Henle)	1.5	.18	.16
Human leukemia (Osgood #111)	1.6	.17	.13
#39 "liver" (Chang)	1.6	.19	.18
KB (Eagle)	1.6	.18	.15
Human conjunctiva (Chang)	1.7	.18	.17
"Liver" (Henle)	1.8	.19	.14

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† Available from the Fisher Scientific Co.

† Round 19 x 105 mm tube, 5.5 min ml capacity.

TABLE II. Progressive Growth of 3 Cell Strains as Measured by Protein Determinations and by Nuclei Counts.

Cell line			Days after planting culture 1	2	3	4
Human leukemia (Osgood #111)	Pd[‡]	μg ± S.D.[*]	784 ± 16	1217 ± 12	1694 ± 11	2520 ± 43
		V[†]	2%	1%	.64%	1.7%
	Cc[‡]	$\times 10^4$[*]	97 ± 6.6	144 ± 4.5	217 ± 9.3	391 ± 42
		V	6.8%	3.1%	4.3%	11%
	Ratio of protein, μg /Cc $\times 10^4$		8.1	8.5	7.8	6.4
HeLa (Gey)	Pd	μg ± S.D.	746 ± 19	1025 ± 53	1498 ± 60	1988 ± 159
		V	2.5%	5.2%	4%	8.0%
	Cc	$\times 10^4$	80 ± 5.8	101 ± 16	163 ± 15	267 ± 39
		V	7.2%	16%	9.2%	14%
	Ratio of protein, μg /Cc $\times 10^4$		9.3	10.1	9.2	7.4
Intestinal epithelium (Henle)	Pd	μg ± S.D.	454 ± 11	743 ± 39	1005 ± 34	1607 ± 176
		V	2.4%	5.3%	3.3%	11%
	Cc	$\times 10^4$	51 ± 4.2	77 ± 8.8	101 ± 12	204 ± 18
		V	8.1%	12%	12%	8.8%
	Ratio of protein, μg /Cc $\times 10^4$		8.9	9.6	10.0	7.9

[*] Mean of 4 replicate flasks ± stand. dev. (σ). [†] Pearson's coefficient of variation $\frac{\sigma \times 100}{\text{mean}}$. [‡] Pd = Protein determination; Cc = Cell count.

sorption curve was a straight line function of the amount of cells up to an optical density $\left(\log \frac{I_o}{I}\right)$ of 0.4.[†] For a given cell type, the bovine albumin equivalent as measured colorimetrically may be converted to cell count, mg dry weight, total nitrogen, protein nitrogen, or any other desired criterion, by appropriate determinations on test suspensions simultaneously measured by the method here described. Some of these conversion factors are indicated for 8 cell types in Table I.

An experiment to show the reliability of the protein determination as a measure of cell growth with 3 different cell lines is summarized in Table II. A number of replicate T-15 flasks were inoculated with each strain, and their protein content and cell count measured daily. The coefficient of variation in the protein content of 4 flasks varied from 1 to 11%, averaging 3%. The coefficient of variation in the cell count procedure varied from 3 to 16%, averaging 9.5%. With each cell line the ratio of the protein content to cell count remained essentially constant until the 4th day, when there was a smaller amount of protein per cell, presumably indicative of a smaller average cell size.

Summary. An analytical method for the measurement of cell growth in tissue culture is described, based on the Lowry method for the determination of protein, and employing a phenol reagent (Folin-Ciocalteau) for color development. The results, referred to a bovine serum albumin standard, may be converted to dry weight, nitrogen or cell count by appropriate conversion factors. Those factors are here given for 7 human cell lines and 1 mouse line.

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Glycogen Stores in Glucagon-Treated Rats. I. Time Factors.* (22246)

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Glucagon is believed to cause hyperglycemia by promoting glycogenolysis in the liver. This is based on the following evidence which has been reviewed in detail recently (1) and which is largely indirect: 1) the hyperglycemic response to glucagon is decreased or absent in fasting, untreated diabetes, severe liver disease(2) and after evisceration (3) when the amount of liver glycogen is presumably low or absent; 2) glucagon is more effective when injected into the portal vein than by any other route of administration and is ineffective after occlusion of the liver circulation(2); 3) glucagon causes a sharp increase in splanchnic glucose production, as measured by venous catheterization in man (4); 4) destruction of the pancreatic alpha cells, presumably accompanied by the liberation of preformed glucagon, causes an immediate hyperglycemia with a decrease in liver glycogen(5); 5) glucagon promotes liver glycogenolysis *in vitro* by activating the phosphorylase system(6). Although these findings would lead one to expect a decrease of liver glycogen in intact animals treated with glucagon, this effect has not been clearly demonstrated. Bürger and Kramer(2) first reported a decrease in liver glycogen in dogs treated with glucagon. However, these pioneering results were obtained with crude preparations of glucagon heavily contaminated with insulin. Furthermore liver glycogen was determined in samples obtained by means of repeated biopsies, a traumatizing technic which may cause glycogenolysis in itself. Similarly, using an impure preparation of glucagon, Heard *et al.*(7) obtained a decrease in liver glycogen in normal rats, while Milman *et al.* (8) obtained similar results in vit. E-deficient rats. In contrast with these findings, the liver glycogen was found normal in rats chronically treated with small doses of glucagon(9), and elevated in animals treated with much larger doses(10).

The reasons for these unexpected results are not clear. It has been suggested(9) that since the glycogenolytic effect of small doses of glucagon is of short duration, the glucose liberated can be redeposited rapidly as liver glycogen, perhaps as a result of increased insulin secretion(11) or through a reversal of the phosphorylase reaction(10). Reports on the effect of glucagon on muscle glycogen are also contradictory and although glucagon does not stimulate muscle phosphorylase activity(6), it appears to inhibit the glycostatic effect of insulin in normal muscles(12,13) and to have a glycostatic effect of its own in the muscles of vit. E-deficient rats(8).

The purpose of this work was to investigate the effects of glucagon and of epinephrine *in vivo* on liver and muscle glycogen and on adrenal ascorbic acid. The latter was determined in order to investigate the possibility that glucagon may stimulate the pituitary-adrenal system as suggested by measurement of corticoid excretion(14) and by analogy with the effects of epinephrine(15).

Methods. Two hundred thirty-one adult Sprague-Dawley albino rats of both sexes were used.§ The animals, kept on a stock diet of Purina checkers, were allowed to eat *ad libitum* or were fasted for 24 hours, as indicated in the tables. Glucagon (Eli Lilly and Co., lot no. 208-158B-197‖; 0.5, 2.5 or 5.0 mg/kg) and epinephrine (0.15 mg/kg) were injected intraperitoneally. In addition, some of the fasted animals received simultaneous intraperitoneal injection of glucose (1 g/kg). After 2, 6, 12, or 24 hours, the animals were decapitated, both adrenal

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§ Rats were a gift of Dr. G. Van Arman of G. D. Searle and Co.

‖ Glucagon was a gift of Dr. O. K. Behrens of the Lilly Research Laboratories.

TABLE I. Effect of Glucagon on Liver and Muscle Glycogen and on Adrenal Ascorbic Acid (mg/100 g of Fresh Tissue).

					Glycogen				Adrenal ascorbic acid	
					Liver		Muscle			
No. of rats	Time after inj., hr	Fasting time, hr	Glucagon, mg/kg	Glucose, g/kg	Avg ± S.E.	% change	Avg ± S.E.	% change	Avg ± S.E.	% change
6	2		—		1884 ± 162		—		431 ± 24	
6	"		0.5		1697 ± 270	− 6	—		342 ± 32	−21
6	"		2.5		624 ± 119	− 67*	—		361 ± 39	−16
6	"		5		591 ± 114	− 69*	—		401 ± 15	− 7
9	"		—		4139 ± 541		467 ± 28		378 ± 17	
20	"		5		1349 ± 188	− 68*	530 ± 22	+13*	358 ± 9	− 6
6	"	24	—		—		—		379 ± 15	
6	"	"	0.1		—		—		312 ± 14	−18
6	"	"	5		—		—		260 ± 34	−31*
6	"	"	—		—		742 ± 63		—	
6	"	"	5		—		753 ± 47	+1.5	—	
5	"	"	—		579 ± 80		—		391 ± 11	
5	"	"	2.5		190 ± 35	− 67*	—		389 ± 16	− 1
6	"	"	—		459 ± 16		395 ± 30		359 ± 5	
6	"	"	5		30 ± 3	− 94*	408 ± 24	+3	262 ± 12	−34*
6	"	"	—		438 ± 85		320 ± 25		346 ± 6	
6	"	"	5		47 ± 7	− 90*	409 ± 9	+27*	256 ± 13	−26*
8	6	"	—	1	517 ± 47		487 ± 47		366 ± 19	
8	"	"	5	1	288 ± 55	− 56*	485 ± 12	−0.4	320 ± 6	−13
5	12	"	—	1	317 ± 34		—		374 ± 21	
5	"	"	2.5	1	164 ± 15	− 48*	—		376 ± 11	+ 1
8	"	"	—	1	1086 ± 26		496 ± 42		308 ± 26	
8	"	"	5	1	822 ± 14	− 25*	552 ± 29	+11	350 ± 11	+13
8	24	"	—	1	54 ± 9		419 ± 35		376 ± 29	
8	"	"	2.5	1	196 ± 33	+264*	387 ± 22	−8	388 ± 10	+ 3
8	"	"	—	1	175 ± 31		349 ± 23		335 ± 15	
8	"	"	5	1	366 ± 25	+108*	487 ± 40	+39*	353 ± 12	+ 5
8	"	"	—	1	125 ± 34		290 ± 39			
8	"	"	5	1	405 ± 64	+224*	345 ± 6	+18		
6	"	"	—	1			909 ± 56			
6	"	"	5	1			879 ± 111	−3.3		

* $P \leqq 0.05$.

TABLE II. Effect of Epinephrine on Liver and Muscle Glycogen and on Adrenal Ascorbic Acid (mg/100 g of Fresh Tissue).

				Glycogen				Adrenal ascorbic acid	
				Liver		Muscle			
No. of rats	Time after inj.	Fasting time, hr	Epinephrine, mg/kg	Avg ± S.E.	% change	Avg ± S.E.	% change	Avg ± S.E.	% change
6	20 min.	24	—	506 ± 27		377 ± 21		397 ± 37	
6	"	"	.15	144 ± 23	– 72*	267 ± 17	–29*	252 ± 18	–37*
6	2 hr	"	—	211 ± 12		301 ± 26		351 ± 25	
6	"	"	.15	538 ± 36	+153*	162 ± 27	–47*	233 ± 20	–49*
6	"	—	—	1082 ± 121		339 ± 20		395 ± 20	
6	"	—	.15	1565 ± 244	+ 44	247 ± 29	–28	301 ± 28	–24*

* $P \overline{<} 0.05$.

glands and samples of liver and of gastrocnemius muscle were removed as rapidly as possible. Liver and muscle were dropped in tared centrifuge tubes containing 30% KOH and their glycogen content was determined according to the method of Good, Kramer, and Somogyi(16) supplemented by the method of Nelson(17). The adrenal ascorbic acid was determined according to the method of Roe and Kuether(18). Each group of rats treated with glucagon or epinephrine had its own control group of rats treated with saline. The statistical significance of the difference between the mean experimental and control values was calculated according to Fisher(19).

Results. The results are presented in Tables I and II. Table I indicates that: 1) 2 and 6 hours after the injection of glucagon there was a marked decrease in liver glycogen which was still noticeable 12 hours thereafter. This decrease appeared to have been greater in animals receiving larger doses of glucagon, and occurred in fed, as well as in fasted animals, whether receiving glucose or not. The lowest value reached was 28 mg 100 g of liver. Similar values of about 30 mg/100 g of fresh tissue were found in 12 animals. 2) Twenty-four hours after the injection of glucagon (2.5 or 5.0 mg/kg) there was a marked and significant increase in liver glycogen. 3) The injection of glucagon was followed by a small and inconstant increase in muscle glycogen. This was statistically significant only in three cases. 4) Two hours after the injection of the largest dose of glucagon (5.0 mg/kg), there was a decrease in adrenal ascorbic acid. This decrease occurred only in fasted animals in which liver glycogen reached very low levels and not in animals which had been fed or had received an injection of glucose. No decrease in adrenal ascorbic acid was found in animals killed 6, 12, or 24 hours after glucagon injection. Table II indicates that 20 minutes after the injection of epinephrine, liver glycogen, muscle glycogen and adrenal ascorbic acid were significantly reduced. Two hours after the injection, muscle glycogen and adrenal ascorbic acid were still below normal, while liver glycogen had increased to a value greater than that of the controls.

Discussion. The results demonstrate that the first effect of glucagon in all doses used is liver glycogenolysis. The absolute magnitude of this effect appears to be greater in animals receiving larger doses of glucagon and in fed animals having more liver glycogen, although the largest per cent decrease (about 90%) was found in animals the liver glycogen of which had been depleted by fasting. This decrease in liver glycogen tends to disappear after 12 hours and is followed 24 hours later by a marked increase. The reasons for this phenomenon are not clear. It has been suggested that, after a period of marked glycogenolysis, glucagon may cause an inversion of the phosphorylase reaction, however, glycostatic effect of glucagon never has been observed *in vivo*. In addition, much smaller doses of glucagon which cause hyperglycemia and therefore presumably stimulate the phosphorylase reaction *in vivo*, do not promote secondary glycogenesis(9). It may be suggested that the primary effect of glucagon, regardless of the dose, is an acceleration of liver glycogenolysis and that the secondary increase in liver glycogen is the result of an increase in insulin secretion due to the accompanying hyperglycemia(20). In addition, the increase of liver glycogen may be favored by the release of ACTH and/or adrenal cortical hormone due to a stress-like effect of the primary glycogen depletion or of large nonphysiologic doses of glucagon. This hypothesis is supported by the following observations: 1) glucagon hyperglycemia stimulates insulin secretion(11), and 2) large doses of glucagon cause a marked decrease in adrenal ascorbic acid (Table I). The effect of the adrenals appears to be a permissive one only, since glucagon (2.5 mg/kg) may cause increase in liver glycogen without significant changes in adrenal ascorbic acid. In this respect, the action of glucagon would be similar to that of epinephrine which also causes: 1) hyperglycemia and, presumably, insulin secretion, 2) decrease in adrenal ascorbic acid, and 3) secondary increase of liver glycogen(15). In the case of epinephrine this compensatory response is very rapid and its effects are seen after 2 hours. In the case of glucagon the glycogenolytic effect disappears only after 12 to 24 hours, perhaps because large doses of glucagon are destroyed less rapidly than epinephrine, or perhaps because the effect of epinephrine on the adrenal cortex is more specific. In agreement with the observation that glucagon does not influence muscle phosphorylase, glucagon did not cause a decrease in muscle glycogen. The slight increase in muscle glycogen observed in these experiments and in those of others(8), may be not a direct and specific effect of glucagon, but a result of the secondary secretion of insulin. This, apparently, cannot overcome the glycogenolytic effect of epinephrine in muscle (Table II). Further experiments are in progress to investigate the role of adrenals, pancreas and pituitary in the glycostatic effects of glucagon.

Summary and conclusions. 1. Effects of epinephrine and various doses of glucagon on liver and muscle glycogen and on adrenal ascorbic acid were studied. 2. Twenty minutes after injection of epinephrine a decrease in liver and muscle glycogen, and in adrenal ascorbic acid were observed. Two hours after injection, liver glycogen was significantly higher than in controls, while muscle glycogen and adrenal ascorbic acid were still below normal. 3. Glucagon caused immediate decrease in liver glycogen. When large doses were used this decrease was followed by secondary increase to values greater than normal. 4. The glycostatic effect of large doses of glucagon in the liver was probably associated with an earlier decrease in adrenal ascorbic acid. 5. Glucagon caused an occasional slight increase of muscle glycogen. 6. It is suggested that the primary effect of epinephrine and of glucagon in all doses is an acceleration of liver glycogenolysis and that their glycostatic effects are due to secondary increases in secretion of insulin and/or adrenal cortical hormones.

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Abnormalities of Urogenital System in Strain A x C Line 9935 Rats. (22247)

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At autopsy congenital absence of one kidney was observed in a rat of the strain A x C line 9935 colony maintained at the National Cancer Institute. Subsequently, a number of rats of this strain were observed to have one kidney missing, and a number were observed to have other urogenital abnormalities. Observations on the extent and incidence of these abnormal conditions are presented herein.

Materials and methods. The strain A x C line 9935 (black agouti irish) rats were obtained from Dr. W. F. Dunning* of the College of Medicine, Wayne University in October 1945. At that time the strain had been inbred by brother x sister matings for 30 generations. The strain A x C line 9935 was started in 1926 by Doctors Dunning and M. R. Curtis from a cross between the August line 1561 and the Copenhagen line 2331. Neither of these 2 strains has ever been observed to have any urogenital abnormality. Dr. Dunning indicated that an abnormality was first observed in March 1937 in animals of the 16th brother by sister generation of the subline (which is the one on which the present observations were made). The right testicle was absent in 5 of a group of 50 males in which methylcholanthrene was being injected. She has observed urogenital abnormalities in the hybrid progeny of a male of the 12th generation of this same subline outcrossed to an August line female. In 1946, she briefly described the urogenital anomaly as occurring with about equal frequency in the 2 sexes and varying in extent in them. Either the right or the left side might be involved. In affected females, an adrenal and an ovary were absent occasionally and in affected males, the testis was absent or atrophic more frequently(1). Following the initial observation in this laboratory a routine search for similar abnormalities was made in young animals (one to 18 days of age) and in adult animals (2 to 17 months of age) of inbred generations 30 through 34. The majority of the adult animals were breeding females and for them data on the size and number of litters produced were available for comparison in the normal and abnormal animals. When the strain A x C line 9935 rats, in which the abnormalities had been observed, were outcrossed to strain M-520, the abnormalities were observed in the F_1 progeny(2).

Results. The incidence of urogenital abnormalities in young and adult rats in shown in Table I. It will be observed that the incidence of urogenital abnormalities in older animals was slightly higher than that in younger animals. However, when the data were analyzed statistically this difference was not found to be significant in either the females or the males (for the females, $\chi^2 = 3.39$; $P = 0.07$, and for the males, $\chi^2 = 0.26$; P is between 0.5 and 0.7).

Animals with urogenital abnormalities always showed either the absence of one kidney or the presence of one cystic kidney. Each of the two manifestations was often associated with various other abnormal conditions. There was no significant difference in the frequency

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TABLE I. Incidence of Urogenital Abnormalities in Strain A × C Line 9935 Rats.

				No. of animals with specific organs abnormal							
Age	Sex	Total	% abnormal	Kidney	Ureter	Oviduct & horn of uterus	Ovary	Ovarian capsule	Testis	Epididymis & vas deferens	Vesicular gland
1-18 days	♀	748	17.9	134	110	99	5				
”	♂	813	16.9	137	95				20		
2-17 mo	♀	189	23.8	45	40	38		18			
”	♂	62	19.4	12	12				5	12	6

of the abnormalities of the kidneys between females and males ($\chi^2 = 1.3$; P is between 0.20 and 0.30). However, they occurred more often on the right side than on the left (182 right and 142 left).[†] The absence of a kidney was seen more frequently on the right side, (150 right and 67 left) and the cystic kidney was seen more frequently on the left (32 right and 75 left).

Among the 45 abnormal adult females there were 33 which lacked one kidney, the right one in 21 and the left in 12. There was no ureter in 26 cases, no uterine horn in 28, no ovarian capsule in 17, and no oviduct in 2. There was hypertrophy of the remaining kidney[‡] in 10, an incomplete ureter in 3, and a cystic and incomplete uterine horn in 4. Twelve of the 45 females had a cystic kidney (3 right and 9 left). The ureter was cystic in 9 cases, the uterine horn incomplete in 3, the ovarian capsule cystic in one, and the opposite kidney hypertrophied in one. The ureter was missing in 2 cases and the uterine horn in one.

In the group of 134 abnormal young females, 91 lacked one kidney, the right one in 65 and the left in 26. The ureter was missing in 84 cases, the uterine horn in 81, and the ovary in 5. The remaining kidney and ureter were cystic in 2 cases, the remaining kidney was hypertrophied in one and cystic in one, and the uterine horn was incomplete in one. There was one animal in which both kidneys, ureters, and uterine horns were lacking. One kidney was cystic in 41 (11 right and 30 left) of the 134 females and both were cystic in one. The ureter was cystic in 25 and the uterine horn in one. The uterine horn was incomplete in 16 cases.

Among the 12 abnormal adult males, there were 8 which lacked one kidney (5 right and 3 left). The ureter was missing in 8, the epididymis in 5, the vas deferens in 6, and the vesicular gland in 4. Atrophy of the testis was observed in 5 cases (the testis of one of these was undescended), of the vesicular gland in 2, and of the epididymis in one. The opposite kidney was hypertrophied in 2 cases. One kidney of 4 (one right and 3 left) of the 12 abnormal males was cystic. The ureter was cystic in 3 cases and ended blindly in one.

Among the 137 abnormal young males, 85 lacked a kidney (59 right and 26 left) and one lacked both kidneys. The ureter was missing in 61 and the testis was undescended in 6. The remaining kidney was hypertrophied in 4 and cystic in 5, and the remaining ureter was cystic in 2. Fifty of the 137 abnormal young males had one cystic kidney (17 right and 33 left). The ureter was cystic in 33 and incomplete in one, and the testis was undescended in 14. There was one animal in which one kidney was atrophied and the testis undescended.

As previously mentioned, a large number of the adult animals used in this experiment were breeding females. Therefore, data on the sizes of the litters of normal and abnormal rats were available. A reduction in the average litter size of successive litters in animals, in which abnormalities were present, as compared with the average litter size of normal animals has been observed (Table II).

[†] These figures do not include 3 cases where either both kidneys were missing or were cystic, and 1 case where the kidney was atrophic.

[‡] Kidneys of small groups of normal animals and of animals lacking one kidney were weighed on spiral spring balance of the Roller Smith Type. Weights (average weight of both kidneys in normal females was 0.87 g and in normal males was 1.1 g while that of one kidney in abnormal females was 1.4 g and in abnormal males was 2 g) confirmed the gross observation of hypertrophy of the remaining kidney.

TABLE II. Average Litter Size for Normal and Abnormal Strain A × C Line 9935 Female Rats.

Group	No. of animals	Avg litter size for successive litters 1	2	3	4	5	6	7	8	9	10
Normal	127	6.9	7.9	7.2	7.0	6.4	6.3	5.8	6.4	4.9	7.0
Abnormal	39	4.6	4.6	4.4	3.7	4.5	4.0	3.8	4.0	1.0	

Discussion. Urogenital abnormalities have been observed in animals by various workers. Hain and Robertson(3,4) observed anomalies, similar to those just described, in Wistar rats and in hybrids between the Wistar strain and a hooded variety. The anomalies were manifested in various ways similar to those described for the strain A x C line 9935 rats, but differed from them in that the abnormalities occurred only on the left side. In contrast to this observation, Faulconer(5) reported a case of hydronephrosis with accompanying abnormalities on the right side in a young female rat of the McCollum hooded strain.

Following small doses of X-rays in mice, Bagg(6-9) observed abnormalities of the viscera, principally of the kidney. These ranged from partial to more pronounced atrophy of one kidney, to absence of one or both kidneys. Hydronephrotic or polycystic kidneys were also observed. The abnormalities occurred as often on the right side as on the left and in females as often as in males. In the present study the findings agreed with this sex distribution, however, the abnormalities occurred more frequently on the right side than on the left.

Summary. The occurrence of congenital abnormalities of the urogenital system of strain A x C line 9935 rats has been described. The principal manifestations were the absence of one kidney or the presence of one cystic kidney. These were accompanied generally by other abnormal conditions. In females, they included a missing ureter, uterine horn, oviduct, ovarian capsule, or ovary; an incomplete ureter or uterine horn; a cystic ureter, uterine horn, or ovarian capsule; or hypertrophy of the remaining kidney, with variation in degree of abnormality. In males, they included a missing ureter, epididymis, vas deferens, or vesicular gland; atrophy of the testis, vesicular gland, or epididymis; cystic ureter; undescended testis; and hypertrophy of the remaining kidney, all also varying in degree.

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Coenzyme A Changes in Liver, Spleen and Kidney of Rats with Infections of *Plasmodium berghei*. (22248)

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Previous studies(1) by one of us have shown that survival *in vitro* of the avian malarial parasite *Plasmodium lophurae* was prolonged when concentrates of coenzyme A (Co A) were added to culture media containing the extracellular organisms. Further work (2) has indicated that the parasites were able to accumulate the coenzyme but were unable to synthesize it. In addition, the concentration of Co A in infected chicken livers was approximately 40% lower than in controls, whereas in ducks the drop in concentration of Co A was not so marked. It appeared, therefore, that the depletion of Co A in the liver was correlated with the accumulation of this essential growth factor in the parasites, and in turn was related to the pathology of infection. The present study was undertaken to determine whether a similar situation existed in a mammalian host infected with a malarial parasite. For this purpose observations have been made on the changes in Co A content of livers, spleens, and kidneys of rats during the course of infection with *Plasmodium berghei*.

Materials and methods. The parasite. The Kisonga strain of *P. berghei* was used in this study. This parasite was obtained from Dr. I. H. Vincke and for the past 2 years has been passed biweekly in this laboratory in 40-60 g rats, from the colony of Dr. John B. Nelson, by the intraperitoneal inoculation of whole blood. *The host.* For experimental purposes Sprague-Dawley rats were used. Of 30 animals 25 days old and weighing an average of 48 g, 15 were infected and the remainder kept as controls. Another 30 animals, 116 days old, weighing an average of 204 g, were treated in a similar fashion. To initiate infection each animal was inoculated with 50,000 parasites intravenously. Thereafter, films were taken daily of tail venous blood in order to follow the parasitemia. Infected rats were fed Purina dog checkers and water *ad libitum*. The amount of food and water consumed by each rat was carefully measured every day and averaged for each of the 2 infected groups. The average amount of food and water consumed was then offered to corresponding control animals. Young infected animals usually refused to eat after the first week of infection while older rats appeared to have an unaffected appetite. Restricted dietary intake of control animals was undertaken in order to rule out any possible effects of starvation on tissue Co A content. *The assay.* At 4-day intervals 3 animals from each infected group and 3 from each control group were sacrificed by decapitation. Each rat was allowed to bleed out. An abdominal incision was made and the spleen, liver, and kidney were removed in that order. Immediately upon removal each organ was weighed, placed in a celluloid tube, and then quick-frozen by immersion in a mixture of alcohol and dry-ice. Tissues so frozen were stored in the deep freeze at –25°C until they could be treated for incorporation in the assay medium. Some tissues were stored in this manner for almost 2 months. To prepare for assay each was ground with 4 times its weight by volume of water in a glass homogenizer in an ice water bath. The homogenate was immediately placed in a bath of boiling water for 10 minutes and centrifuged. Co A was determined in the supernatant by microbiological assay, using the methods cited in the work with *P. lophurae*(2).

Results. Essential host-parasite data are described in Table I. In each of the 2 age groups parasitemia followed a course similar to that reported elsewhere(3). In addition changes in the ratio of organ to body weight from the 4th to the 12th days of infection are presented. In young rats the spleen to body weight ratio increased 850% over controls by the 12th day of infection, whereas in old animals the increase was about 600%. At the

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TABLE I. Host-Parasite Data.

Group	No.	Day of infection sacrificed	Age, days	Wt†	Parasites,† 10000 rbc	Ratio liver to body wt*	Ratio spleen to body wt*	Ratio kidney to body wt*
1 X	3	4	29	67	706	.053	.0095	.0062
2 X	3	8	33	94	3410	.067	.028	.0059
3 X	3	12	37	80	6520	.062	.036	.0059
4 X 13	1	16	41	96	<1	.052	.034	.0049
14	1	16		57	12600	.063	.022	.0086
1 Control	3		30	61		.041	.0038	.0053
2 "	3		34	86		.040	.0050	.0049
3 "	3		38	86		.037	.0031	.0046
4 "	2		42	86		.036	.0026	.0041
5 X	3	4	116	208	30	.032	.0043	.0035
6 X	3	8	120	208	550	.039	.012	.0034
7 X	3	12	124	212	720	.037	.017	.0035
8 X	3	16	128	218	1	.034	.013	.0036
9 X	3	20	132	223	<1	.035	.0090	.0037
5 Control	3		117	200		.028	.0027	.0037
6 "	3		121	203		.026	.0025	.0035
7 "	3		125	212		.029	.0021	.0035
8 "	3		129	211		.026	.0023	.0037
9 "	3		133	223		.031	.0024	.0036

* Avg. † Avg for surviving animals.

same time, the liver to body weight ratio in young animals was 63% greater, and one half that value in old animals. The kidney to body weight ratio showed an increase of 25% in the young animals and no change in old rats.

Co A content of liver, spleen, and kidney at various stages during the course of infection is described in Table II. In the livers of young rats, a drop in Co A concentration was evident by the 8th day of infection. The total Co A present remained about the same even though liver to body weight ratios were 50% greater than in controls. The average percentages dry weight for the livers of 4 infected animals and 4 control animals sacrificed on the 8th day of infection were respectively 25.6% and 28.7% indicating that the drop in concentration of Co A was not simply dilution due to higher water content. In the older animals neither the decrease in coenzyme concentration nor the increase in liver size was so marked. In the spleen, on the other hand, the concentration of Co A remained constant through the course of infection, while the total amount present was proportional to the size of the organ. The kidneys of young rats showed a slight drop in Co A concentration with no change in the total, while in the older animals no changes were apparent in either organ size or Co A content.

It should be mentioned here that no differences were noted in concentrations of free pantothenate found in the livers or spleens of experimental and control animals. In both young and old groups the range for liver was 4-10 μg per g tissue. In spleen the range for young animals was 2-3 μg per g and in the older groups 1-3 μg per g. In the kidneys, both infected and uninfected old rats showed a range of 19-26 μg/g, whereas infected young rats had concentrations of 6-11 μg/g as contrasted to 12-28 μg/g in uninfected young animals.

Discussion. Changes observed in liver Co A of rats infected with *P. berghei* were similar to those described for ducks with infections of *P. lophurae*. In both cases there was a marked drop in the concentration of Co A, but the concomitant enlargement of the liver resulted in a total Co A content approximating that of controls. However, in chickens infected with *P. lophurae*, both the concentration and total amount of Co A in the liver were reduced. It is of interest to note the results observed with rats 13 and 14 in group 4 X (Table II). Rat 14, sacrificed at the terminal stages of infection, showed a decrease in both concentration and total Co A,

TABLE II. Average Units of Coenzyme A

		Liver		Spleen		Kidney	
Group	Category*	Per g†	Per organ	Per g†	Per organ	Per g†	Per organ
1 X	Infected—Young	163	580	20	13	55	24
2 X		98	594	21	54	56	30
3 X		103	512	21	60	51	24
4 X 13		157	790	24	78	67	32
14		81	301	19	24	58	29
1 C	Restricted diet controls —Young	192	486	20	5	67	22
2 C		174	596	18	8	68	29
3 C		162	522	18	5	72	28
4 C		172	509	19	5	53	19
5 X	Infected—Old	112	737	19	18	59	44
6 X		110	851	17	54	36	27
7 X		110	848	23	69	47	34
8 X		109	813	23	68	54	42
9 X		93	728	20	39	74	60
5 C	Restricted diet controls —Old	129	713	15	8	54	40
6 C		135	704	14	7	49	35
7 C		151	913	21	9	50	37
8 C		126	701	17	9	60	46
9 C		112	760	18	9	47	38

* For more complete data on course of infection see Table I. † Fresh wt.

despite its much enlarged liver. Rat 13, killed at the same time, was well on its way to recovery. Its liver had a normal concentration of Co A, but was still considerably enlarged, so that the total Co A was above that seen in controls.

The short period of reduced food intake which accompanied the infection in young animals, and which was artificially produced in the corresponding uninfected group, evidently had little, if any, effect on the Co A concentration of the liver. Average Co A concentration in the 6 livers of rat groups sacrificed on the 8th and 12th day, at the height of infection, showed a large decrease in infected animals and values for controls intermediate to those reported in the literature. Livers of young infected rats contained 100 units of Co A per g and mature infected rats 110 units. Of the controls the young animals showed 168 units, whereas the older ones had 143 units per g liver. The disparity among values cited in the literature(4-6) and with these might be due to the different strains of rats used.

Kidneys of rats infected with *P. berghei* exhibited no appreciable changes in either the concentration or total Co A present. However, there was a marked difference in the concentration of free pantothenate in the kidneys of young infected and control animals. This may have resulted from increased reabsorption of free pantothenate in the infected group because of the heavy demands of the parasite on the host for Co A.

In contrast to the liver, Co A concentration in spleen remained constant whereas the total amount present was proportional to increase in size of the organ. This suggests that the cells involved in hyperplasia of the spleen, in response to the infection, have the same Co A content as those originally present, and is in accordance with the finding that spleens of chickens with infections of *Plasmodium gallinaceum* showed no changes in glucose metabolism(7).

In view of both present and past work, it seems that the ability to produce Co A may rank as one of those factors of innate immunity which determine the ultimate death or survival of the host. When the demands of the parasite are so great that the host cannot supply its own tissues with sufficient Co A, the animal succumbs, whereas the animal will survive if it can maintain the coenzyme supply until the forces of acquired immunity control the infection. In addition the decreased concentration of Co A in the livers of infected animals might be responsible for the decreased ability of that organ to convert glucose to glycogen(8). In any case, the observed changes are in harmony with the hypothesis that in mammalian as well as in avian malaria, disturbance in Co A metabolism is significant in the pathogenic effects of the infection.

Summary. Co A concentration and total content in liver, spleen, and kidney of normal rats and rats with infections of *Plasmodium berghei* have been described. During the course of infection the concentration of Co A in the liver dropped but the total amount present was unchanged. In the spleen the concentration remained static, whereas the total was proportional to the increased size of the organ. The kidney showed no appreciable changes in Co A, but a decreased concentration of free pantothenate was apparent in young infected animals.

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Inhibition of Mumps and Influenza B Virus Multiplication by Synthetic Poly-D-Lysine.* (22249)

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It has been previously reported that the synthetic basic polypeptide, poly-L-lysine, protects the chick embryo for a period following inoculation with a number of animal viruses such as mumps virus(1), infectious bronchitis, Newcastle disease viruses(2), and influenza B virus(3). A comparative study using a synthetic basic polyelectrolyte, as polyvinylamine, showed that this polymer was not as effective a virus inhibitor as poly-L-lysine and was almost 5 times more toxic to the chick embryo(3). Proteolytic enzymes (4) in the chick embryo have been demonstrated to destroy poly-L-lysine injected into the allantoic fluid(5). Since this enzymatic destruction of the L-polypeptide might be a factor that limits its protective action against viruses and as D-polypeptides are usually less susceptible to enzymatic hydrolysis, we have studied the effectiveness of poly-D-lysine against mumps and influenza B viruses in the chick embryo.

Materials and methods. The mumps and influenza B Lee strain viruses† were cultivated in chick embryos according to a previously described procedure(1,3). The virus cultures and the procedures for inoculation, harvesting and estimation of the extent of virus multiplication by hemagglutination titrations, were similar to those used by Green and Stahmann(1,3). In all experiments fertile White Leghorn eggs were employed. Poly-D- and poly-L-lysine hydrochlorides of molecular weights of about 3,900 and 3,600 respectively (determined by end group analysis) were prepared by methods published elsewhere(6-8). The maximum non-lethal dose of poly-D-lysine was approximately 0.1 mg and that of poly-L-lysine was 1.0 mg per chick embryo.

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TABLE I. Reduction of Hemagglutination Titer of Mumps Virus in Allantoic Fluid of Chick Embryo Injected with Polylysine.

1st inj., 0.1 cc intra-allantoic	2nd inj., 0.1 cc intra-allantoic (1 hr later), E.I.D.†	Hemagglutination titer* Individual allantoic fluids					Geom. mean	Log mean	Difference from control log
Saline	10	160	80	160	32	80	88	1.94	
.04 mg DPL‡	”	0	0	0	0	0	0		1.94
” LPL§	”	0	0	0	0	0	0		1.94
Saline	100	256	128	64	256	256	169	2.23	
.04 mg DPL	”	32	0	0	2	8	8	.83	1.40
” LPL	”	0	16	16	2	4	7	.90	1.33
Saline	1000	256	512	256	128	256	256	2.41	
.04 mg DPL	”	32	8	0	0	16	16	1.20	1.21
” LPL	”	64	0	4	8	0	13	1.40	1.31
Saline	10000	256	256	256	256	128	223	2.35	
.04 mg DPL	”	64	16	32	0	16	27	1.43	.92
” LPL	”	64	32	16	64	16	32	1.51	.84

* Expressed as the reciprocal. † E.I.D. = Embryo infectious dose. ‡ Poly-D-lysine of avg molecular wt 3900. § Poly-L-lysine of avg molecular wt 3600.

Results. (a) *Effect of poly-D- and poly-L-lysine on inhibition of mumps virus multiplication in chick embryo.* Data of Table I show comparative effectiveness of poly-D- and poly-L-lysine in their inhibition of mumps virus multiplication. Groups of 5 embryos were injected intraallantoically with 0.04 mg of each polypeptide followed one hour later with 10 to 10000 embryo infectious doses of mumps virus. Controls were injected with saline. After 6 days of incubation at 37°C the eggs were harvested and hemagglutination titers of the individual allantoic fluids determined. It has previously been shown that poly-L-lysine cannot be demonstrated in the allantoic fluid by hemagglutination technic longer than about 24 hours after injection into the allantoic sac of the chick embryo(5), however, poly-D-lysine was demonstrated by the same technic to persist in the embryo up to 36 hours after injection. The data of Table I show that poly-D-lysine is equally as effective in inhibiting mumps virus multiplication as poly-L-lysine. It was thought that a difference in the rate of destruction of poly-D-lysine and poly-L-lysine as it influences the antiviral activity might be shown at lower concentrations of the polypeptides. Table II summarizes the results obtained when 0.001 to 0.04 mg levels of poly-D- and poly-L-lysine were administered to embryos injected with 1000 embryo infectious doses of mumps virus. Although the effectiveness of polylysine had decreased considerably at the 0.001 mg level, the comparative effectiveness of both types of polypeptides remained essentially the same.

TABLE II. Effect of Different Levels of Polylysine on Hemagglutination Titer of Allantoic Fluids Infected with 1000 E.I.D. of Mumps Virus.

1st inj., 0.1 cc intra-allantoic	2nd inj., 0.1 cc intra-allantoic (1 hr later), E.I.D.	Hemagglutination titer of allantoic fluids*	Difference from control log
Saline	1000	256	
.04 mg DPL†	”	16	1.21
” LPL‡	”	13	1.31
Saline	”	256	
.08 mg DPL	”	0	2.41
” LPL	”	4	1.81
Saline	”	256	
.1 mg DPL§	”	0	2.41
” LPL	”	0	2.41
Saline	”	128	
.01 mg DPL	”	8	1.20
” LPL	”	11	1.05
Saline	”	256	
.001 mg DPL	”	38	.83
” LPL	”	32	.90

* Expressed as reciprocal of geometrical mean of hemagglutination titers of allantoic fluids from groups of 5 to 6 embryos obtained 6 days after inoculation.

† Poly-D-lysine of avg molecular wt 3900.

‡ Poly-L-lysine ” ” ” ” 3600.

§ Maximum of one survived of a number of experiments done when inj. with 0.1 mg of DPL plus various levels of virus, probably due to the toxicity level of DPL.

TABLE III. Reduction of Influenza B Multiplication in the Chick Embryo by Polylysine.

1st inj., 0.1 ml intra-allantoic	2nd inj., 0.1 ml intra-allantoic (1 hr later), I.D.$_{50}$	Hemagglutination titer*	Diff. from control log
Saline	10	320	—
.04 mg DPL†	"	8	1.60
" LPL‡	"	5	1.81
Saline	100	640	—
.04 mg DPL	"	22	1.46
" LPL	"	19	1.53
Saline	10000	640	—
.04 mg DPL	"	78	.91
" LPL	"	80	.90

* Expressed as reciprocal of geometrical mean of hemagglutination titers of allantoic fluids from groups of embryos obtained 2 days after inoculation.

† Poly-D-lysine of avg molecular wt 3900.

‡ Poly-L-lysine " " " " 3600.

(b) *Comparative effect of poly-D- and poly-L-lysine on influenza B virus Lee strain.* The data of Table III show the comparison of poly-D- and poly-L-lysine on influenza B virus. The procedures followed were similar to those used with mumps virus with the exception that the incubation period was only 2 days. The results again show that the two polypeptides have approximately the same antiviral activity.

Discussion. These data demonstrate that poly-D-lysine inhibits mumps and influenza B virus multiplication equally as well as poly-L-lysine. The extent of the inhibition obtained by poly-L-lysine was in agreement with that reported by Green and Stahmann (1-3). Since data have been obtained indicating that poly-L-lysine is metabolized in the allantoic fluid(5), it was thought that the duration of the virus inhibitor activity may be limited by the rate of metabolism of the polypeptide. However, the parallel antiviral activity of both the L and D polypeptides would suggest that poly-D-lysine may also be metabolized or hydrolyzed by the enzymes of the allantoic fluid. This metabolism of the D-polypeptide was further indicated by experiments which demonstrated that poly-D-lysine could not be detected in allantoic fluid by its hemagglutination ability for periods greater than about 36 hours following injection of the D-polypeptide. These observations suggested that there was an enzyme whose specificity was such that it allowed hydrolysis of poly-D-lysine. Experiments were therefore undertaken to demonstrate more clearly the existence and action of this new poly-D-lysyl peptidase. In subsequent experiments of this laboratory, it was found that poly-D-lysine is in fact hydrolyzed by extracts of pancreas powder as rapidly as is poly-L-lysine(6).

Summary. Poly-D-lysine, though ten times more toxic, inhibited mumps and influenza B Lee strain viruses to approximately the same extent as poly-L-lysine in the allantoic sac of the chick embryo. As little as 0.001 mg per embryo of either lysine polypeptide was sufficient to inhibit high levels of viral infection. Evidence was obtained which suggested the existence in allantoic fluid of enzymes which hydrolyze both poly-D-lysine and poly-L-lysine.

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Effect of Dietary Carbohydrate on Experimentally Induced Hypercholesteremia and Hyperbetalipoproteinemia in Rats.* (22250)

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The biliary excretion of bile acids by rats is increased when corn starch is used as the dietary carbohydrate in lieu of sucrose(1). A major portion of 4-C^{14} cholesterol administered to the rat or to man is excreted as labeled bile acids in the bile(2,3), and feeding of high levels of cholesterol to rats results in an increased biliary excretion of bile acids(1). Feeding of bile acids with cholesterol to rats results in a marked hypercholesteremia and hyperbetalipoproteinemia(4). There is still some question(5,6), however, as to the principal mechanisms involved. Any factor influencing bile acid excretion might be reflecting variations in conversion of cholesterol to bile acids. The effects of feeding various carbohydrates on experimentally induced hypercholesteremia and hyperbetalipoproteinemia in the rat were, therefore, investigated.

Methods. Rats were male albinos weighing 250 g (range: 240-260 g) and were received from Charles River Laboratories. The animals were individually caged and given water and diet *ad libitum.* There was essentially equal caloric intake in experimental groups and mean weight gains were identical (about 2 g/day). The basal diet consisted of casein 20%, corn oil 8%, carbohydrate 56%, cholesterol‡ 5%, celluflour 5%, salts(7) 4%, vitamins and trace nutrients as previously described(8). In some diets 1.5% cholic acid§ was included. Diets will be designated in the text and Tables according to type of carbohydrate and presence of cholic acid. Rats were fed experimental diets for 28 days. Serum total cholesterol was determined by the method of Abell *et al.*(9); the serum beta-lipoproteins were determined by the method of Gofman *et al.*(10). Liver total cholesterol values were determined by modification of the method of Abell *et al.*(9).

Results. Effect of starch and sucrose feeding on cholate induced hypercholesteremia and hyperbetalipoproteinemia. The results of analyses for serum total cholesterol and beta-lipoprotein concentrations and liver total cholesterol concentrations are indicated in (Exp. 1) Table I. Rats of the starch-cholic groups had lower serum total cholesterol values (mean = 257 mg %) than the sucrose-cholic group (mean = 378 mg %) ($p < 0.01$). The liver total cholesterol concentration was strikingly elevated in both groups but to about the same extent. Approximately two-thirds of the beta-lipoproteins in all the animal patterns had migration rates between S_f 20 and S_f 60 with maximum concentration at approximately S_f 35 and negligible amounts below S_f 12.‖ The mean serum concentration of S_f 20-100 molecules of the starch-cholic group was 216 mg % compared to a mean value of 413 mg % for the sucrose-cholic group ($p < 0.01$). Probably these differences between the 2 dietary groups were related to the previously demonstrated(1) lesser excretion of bile acids by rats fed diets containing sucrose.

Effect of sucrose, fructose, and glucose feeding on cholate induced hypercholesteremia. Serum total cholesterol and serum beta-lipoprotein responses obtained after feeding cholesterol and cholic acid diets containing sucrose, fructose, and glucose for 28 days are

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‡ Cholesterol was furnished by Armour & Co.

§ Cholic acid was furnished by Mr. de Haen, Miles-Ames Research Div., Elkhart, Ind.

‖ Determination of quantity of lipoproteins was based on refractive indices established on pooled human sera.

TABLE I. Effect of Dietary Carbohydrate on Serum and Liver Cholesterol Concentrations and Serum Beta-Lipoprotein Concentrations in Rats.

					Serum β-lipoproteins (mg %)			
Exp.	Diet*	No. of animals	Serum cholesterol, mg %	Liver cholesterol, mg %	S_f 0–11	12–20	20–100	100–400
1	Cholate-sucrose	19	378 ± 23 †	8560	20	67	413 ± 49†	128
	-starch	19	257 ± 12	8420	14	48	216 ± 13	8
2	Cholate-sucrose	10	414 ± 36		23	82	521	60
	-glucose	10	361 ± 29		22	95	417	20
	-fructose	10	411 ± 20		16	56	504	90
3	Sucrose	10	124 ± 4.5					
	Starch	10	106 ± 3.9					
4	Cholate-sucrose	10	380 ± 35					
	-sucrose sulfa‡	8	348 ± 28					
	-starch	8	270 ± 25					
	-starch sulfa	9	361 ± 23					

* Rats were fed for 28 days on diets described in text. Diets contained 5% cholesterol. Presence of cholic acid and type of carbohydrate are indicated in "Diet" column.

† All values are means ± stand. errors of the means.

‡ Sulfasuxidine was added at 0.2% of the diet where "sulfa" is indicated in the "Diet" column.

indicated in (Exp. 2) Table I. Serum cholesterol was elevated in each group. Rats fed glucose had somewhat lower serum cholesterol and beta-lipoprotein levels, although the difference was not of high order of statistical significance. The lower serum cholesterol levels in rats fed a glucose containing diet compared to those fed the sucrose diet corresponded with the previous finding that there was a slightly greater 24-hour biliary excretion of bile acids in the former group(1).

Serum cholesterol response when cholesterol was fed without cholic acid. Serum total cholesterol values obtained after feeding sucrose and starch containing diets with cholesterol but without cholic acid for 28 days are indicated in (Exp. 3) Table I. The sucrose group had a mean value of 124 mg % compared to 106 mg % for the starch group ($p < 0.01$). This finding probably indicates that the starch group has a lower serum cholesterol value due to its greater excretion of intrinsically derived bile acids(1).

Effect of adding sulfasuxidine to cholate containing diets. Variations in intestinal flora could explain the different effects of sucrose and starch on cholesterol-cholate induced hypercholesteremia. Therefore, 4 series of rats were fed the cholesterol and cholate containing diet. Two series were given starch as the dietary carbohydrate, and 2 were given sucrose. One of the starch groups and one of the sucrose groups were fed 0.2% of diet as sulfasuxidine. The cholate-sucrose, cholate-sucrose-sulfa, and cholate-starch-sulfa groups had similar mean serum cholesterol values, whereas the cholate-starch group had a lower ($p = 0.01$) mean serum cholesterol value (Exp. 4) Table I. It would thus seem that the gastrointestinal flora in animals fed a diet containing corn starch might contain some organisms which were influencing the metabolism of cholesterol or its catabolic end product, cholic acid. Norman(11) has shown that administration of chemotherapeutic agents to rats destroys gastrointestinal organisms responsible for hydrolysis of conjugated bile acids.

Thus, in cases where a greater biliary excretion of bile acids (as with feeding of corn starch) had been previously observed(1) a lesser degree of hypercholesteremia was observed when cholesterol or cholesterol and cholic acid were added to the diet. There is an inferred causal relationship between lower excretion of bile acids and increased cholesteremia, although the mechanisms involved and their relationship to dietary carbohydrate have not been established.

Summary. Male albino rats were fed diets for 28 days in which the type of carbohydrate was varied and serum cholesterol and beta-lipoproteins were determined. 1. Rats fed diets containing sucrose as the carbohydrate and with added cholesterol and cholic acid had higher serum cholesterol and serum beta-lipoprotein concentrations than did control rats fed diets with corn starch substituted for sucrose. Liver cholesterol concentrations were not significantly different. 2. Feeding of sucrose, glucose, and fructose as the dietary carbohydrate had essentially equal effects on cholesterol-cholic acid induced hypercholesteremia. 3. Rats which were fed cholesterol containing diets without cholic acid and with corn starch as the carbohydrate had somewhat lower serum cholesterol values than did the controls which were fed sucrose. 4. When sulfasuxidine was added to sucrose containing diets, there was no change in serum cholesterol values; however, addition of sulfasuxidine to starch containing diets resulted in elevation of serum cholesterol values to the level of that in rats fed sucrose.

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Isolation of Partially Purified Human Plasmin (Fibrinolysin). (22251)

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Although many methods for the preparation of plasmin from blood have been described, the utilization of highly purified plasminogen as a starting material appeared to offer the possibility of providing much purer plasmin than any previously reported.

This paper describes the preparation of plasmin from purified plasminogen, and the alcoholic fractionation of the resulting solution to obtain a plasmin product which is stable at low temperature and, in terms of fibrinolytic activity, is far more potent than any previously described fibrinolysin.

Materials and methods. Plasminogen. Lyophilized powder prepared by the method developed in this laboratory(1) from human plasma fraction III[†]. *Streptokinase* (SK). Commercial Varidase (Lederle).[‡] Lots Nos. 7-1089-165A and 7-1089-142A were used. These contained 4,700 and 3,000 SK units per mg, respectively. *Fibrinogen.* Bovine Fraction I (Armour) was reprecipitated three times by the method of Laki(2) and was lyophilized. The critical assays were rechecked with fibrinogen which was reprecipitated seven times to eliminate the possibility that contaminating bo-

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[‡] Kindly supplied to us by Mr. Frank Ablondi of the American Cyanamid Co., Lederle Laboratories Division.

vine plasminogen might have contributed to the results obtained(3,4). The quantity of fibrinogen in the lyophilized powder was calculated from nitrogen determinations, and a suitable amount was dissolved in phosphate buffer to a concentration of 0.4%. Fresh solutions were prepared for each series of assays. *Phosphate buffer.* 0.05 M, pH 7.8 for assays using 3 times purified fibrinogen; 0.01 M, pH 7.8 for 7 times reprecipitated fibrinogen. *Thrombin.* Commercial thrombin (Upjohn) was dissolved in an equal mixture of saline and glycerol to a concentration of 100 units per ml. *Assay of Fibrinolytic Activity.* A slight modification of the method of Ferguson(5) gave reproducible results. In one tube (8x70 mm), 0.5 ml of diluted enzyme solution, 0.5 ml of buffer and 0.04 ml of thrombin solution were mixed. To a second tube were added 0.5 ml of fibrinogen solution and 0.5 ml of buffer. The tubes were mixed immediately by pouring back and forth 3 times, placed in a water bath at 37.5°C, and the time of clot lysis determined. This tube contained a dilution of enzyme solution which had previously been found to dissolve the clot in the range of 270 to 330 seconds and was designated as 100%. Similar tubes containing 0.2 and 0.1 ml of diluted enzyme solution were made up to volume with buffer and were designated as 40 and 20%, respectively.

TABLE I. Alcohol Fractionation of 100 mg of Plasminogen after Streptokinase Activation.

% alc.	Nitrogen, mg/ml	Activity, units/ml (×1000)	Spec. activity, units/mg N (×1000)	Total activity in fraction,* units ($\times 10^6$)
0	.67	40	60	1
10	.29	123	423	.6
21	.21	43.6	207	.2
Ppt. at pH 5.3	.89	4	4.5	.02
0	.85	40	47.06	1
10	.26	86	330.8	.4
0	.84	40	57.5	1
10	.46	70	151	.35
21	.32	21	66	.1
Ppt. at pH 5.3	.97	1.68	1.73	.01
0	.82	40	48.78	1
10	.37	100	294	.5
15	.21	35.6	170	.2
21	.14	17.8	127	.09
0	.76	40	53	1
21	.58	192	330	1
0	1.03	40	39	1
21	.76	155.2	203	.8

* Unfractionated solutions were 25 ml each. 0.5 ml of a 1:200 dilution was used in each assay. Total activity, therefore, was: 100 × 2 × 200 × 25 as described in the text. Alcohol fractions were dissolved in 5 ml of water which was then diluted 1:400 for assay.

Units of Activity. The logarithm of the lysis time in seconds was plotted against the logarithm of the concentration (100, 40 or 20) and a straight line was drawn through the points. For a given fibrinogen solution, the slope of the line was constant. The activity of the 100% tube was arbitrarily assigned the value of 100 units. To obtain the activity per ml of original enzyme solution, the units obtained in each assay were multiplied by 2, since 0.5 ml is used in the assay, and then by the dilution factor. The specific activity is the activity per mg of nitrogen. *Activation.* The plasminogen powder was suspended in distilled water and dissolved by the addition of a few drops of N HCl. The concentration of enzyme was then adjusted to 4 mg/ml by the further addition of water. This solution was adjusted to pH 6.8 by the careful addition of dilute NaOH solution and 5,425 units of streptokinase per mg of plasminogen was added. The mixture was incubated at 37.5°C for 10 minutes after which the solution was adjusted to pH 8.7 and centrifuged. The pH was then lowered to 7.6 and again centrifuged, leaving a clear solution of plasmin. *Alcohol Fractionation.* The plasmin solution was chilled in an ice and salt bath to about 0°C and cold 95% ethyl alcohol was added dropwise with mechanical stirring until the desired alcohol concentration by volume was reached. The precipitate was permitted to settle overnight at 4°C, collected by centrifugation and dissolved in distilled water. A solution of plasmin so obtained has retained its original activity after seven months in the frozen state.

Results. Table I shows the total and specific activities of the activated solutions, of the alcohol fractions, and of the remainder of the active material which precipitated at pH 5.3. It will be observed that the precipitate obtained from 10% alcohol is the most active per mg of nitrogen, and that the fractionation is sharp, little activity remaining in the supernatant solution after removal of the 21% alcohol fraction. The combined activities recovered averaged about 75% of that present in the unfractionated solution, and the specific activity of the combined active fractions was about 3.5 times that of the starting solution.

To insure that these results did not represent the presence or absence of SK in any of the fractions, additional SK, 1000 units per assay, was added to each fraction with no effect upon the activity. In addition, to control SK to plasminogen ratios, 1000 units of SK per mg of enzyme nitrogen was added to each fraction assayed, again without affecting the activity appreciably.

Discussion. Although the plasmin obtained by streptokinase activation of purified plasminogen followed by alcoholic fractionation is the most potent fibrinolytic preparation thus far reported, the total yield and specific activity obtained may possibly be increased by varying the conditions of activation and fractionation. It is recognized that the amount of SK used in this procedure is quite large. In our laboratory, such amounts of SK alone or after mixing with plasminogen have regularly produced severe toxic effects and even death in the dog. Studies in dogs which are being carried out in the laboratory by Messrs. Fishbein and Newman, in collaboration with Dr. Louis Nahum, will be reported in detail elsewhere. Preliminary results indicate that the intravenous infusion of up to 1 mg/kg of purified plasmin is tolerated by the dog with no significant alterations of body temperature, blood pressure or electrocardiogram. Incoagulability of the dogs' blood appeared within 5 minutes of the injection. The following day, clotting times had returned to normal. The absence of toxic reactions in the dog of the new plasmin preparation would indicate that the toxic materials originally present in the SK(6) are largely eliminated by the fractionation procedure. Streptokinase itself is completely soluble in 21% alcohol. It is possible that a non-toxic SK-plasmin complex is the active form of the enzyme.

The dissolving of clots made from seven times reprecipitated fibrinogen would appear to rule out bovine plasminogen as an essential element of the clot-lysing system. The addition of 2 ml of human blood to 0.1 mg of purified plasmin resulted in immediate incoagulability. This experiment also eliminates the possibility that the release of heparin from the liver of the dog was responsible for the incoagulability observed. In preliminary experiments being carried out in association with Dr. W. Glenn the *in vivo* dissolving of a radiochromium labeled clot in the dog has been observed after the intravenous injection of 0.5 mg of plasmin per kg. The dissolving of the clot was confirmed by autopsy.

With the availability of this water-soluble, fibrinolytic enzyme preparation, preliminary clinical studies of the efficacy of this material in thrombotic conditions is now brought much closer. New light may also be thrown upon the physiology of blood coagulation by studies of the action of plasmin. Work is now in progress for the further purification and characterization of this protein.

Summary. A method for preparation and isolation of plasmin from purified human plasminogen after activation with streptokinase is presented. The product is water soluble, stable in the frozen state and produced no toxic manifestations in the dog after intravenous infusion of 1 mg/kg.

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Observations on Bound Ascorbic Acid in Guinea Pig Liver.* (22252)

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(Introduced by J. Murray Steele.)

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Sealock and co-workers(1,2) recently reported the presence of bound L-ascorbic acid in guinea pig liver. In their study, free L-ascorbic acid was extracted from liver with 95% ethanol and bound L-ascorbic acid was liberated from the alcohol-precipitated protein fraction by heating in acid solution. These investigators used the colorimetric method of Roe(3,4) to measure L-ascorbic acid present in the free and bound forms. The availability of L-ascorbic-1-C^{14} acid(5,6) has allowed further study of the presence of bound L-ascorbic acid in guinea pig liver. In addition, radioactive tracer technics have made possible observations on the binding of D-ascorbic acid in guinea pig liver.

Material and methods. L-ascorbic-1-C^{14} acid and D-ascorbic-1-C^{14} acid had specific activities of 1.40 and 1.18 μc per mg respectively. D-ascorbic-1-C^{14} acid was prepared by the method used for L-ascorbic-1-C^{14} acid (7-8) except that D-xylosone was used as the starting material instead of L-xylosone. Preparation of samples and assay for C^{14} were carried out as described previously(5). Male guinea pigs purchased from Rockland Farms, 2 to 3 months old and weighing 250 to 350 g, were used. Carrier dilution experiments(9) have shown that at least 95% of the C^{14} in the liver of guinea pigs 24 hours after intraperitoneal administration of L-ascorbic-1-C^{14} acid is present as L-ascorbic acid. It was therefore possible in the present study to determine the amounts of free and bound L-ascorbic acid by radioactive assay. Three normal guinea pigs and 2 guinea pigs maintained on a vit. C free diet for 14 days each received 3 mg of L-ascorbic-1-C^{14} acid by intraperitoneal injection. Twenty-four hours later the animals were sacrificed and their livers removed. A portion of the liver was homogenized with water and the total C^{14} in the liver was determined on an aliquot of the resulting homogenate by a wet combustion method(5). The remaining liver (5 g) was extracted 6 times with 30 ml portions of 95% ethanol by the procedure employed by Summerwell and Sealock(1). Measurements of C^{14} in the different alcoholic extracts showed that essentially all the free ascorbic acid was recovered in the first 3 extractions. The resulting alcohol-precipitated protein fraction was treated with hot 5% metaphosphoric acid(1) and after centrifugation a suitable aliquot of the supernate was assayed for C^{14} by the wet combustion technic(5). The % of ascorbic acid in the bound form was taken as the ratio of the total amount of C^{14} recovered after heat treatment of the alcohol-precipitated fraction to the total amount of C^{14} present in the liver. Further confirmation of C^{14} in the bound fraction being present as L-ascorbic acid was obtained by a carrier dilution technic(6). 200 mg of non-radioactive L-ascorbic acid was added prior to heat treatment and the C^{14} present in this fraction was recovered as the 2, 4 dinitrophenylosazone derivative of ascorbic acid. This technic has been shown to be highly specific for ascorbic acid. The determination of free and bound D-ascorbic acid in guinea pig liver was carried out in the same way as described for L-ascorbic acid. Two guinea pigs maintained on a vit. C-free diet for 14 days each received 3 mg of D-ascorbic-1-C^{14} acid by intraperitoneal injection and the animals were sacrificed 2 hr later and their livers removed. With D-ascorbic acid it was necessary to carry out the analyses at 2 instead of 24 hours used for L-ascorbic acid. because of the rapidity with which the former

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TABLE I. Bound L-Ascorbic Acid and D-Ascorbic Acid in Guinea Pig Liver, 7 Experiments.

Compound	Animal	% in bound fraction*
L-ascorbic-1-C^{14} acid	Normal guinea pig	14
Idem	*Idem*	23
"	"	20
"	Vit. C deficient guinea pig	31
"	*Idem*	25
D-ascorbic-1-C^{14} acid	"	35
Idem	"	34

* Expressed as % of C^{14} recovered after heating in acid of the alcohol-precipitated protein fraction to the total C^{14} present in the liver.

compound is eliminated by the guinea pig (10). A carrier dilution technic similar to that used for L-ascorbic-1-C^{14} acid showed that all the C^{14} in the liver 2 hours after administration of D-ascorbic-1-C^{14} acid is present as D-ascorbic acid(10).

Results. An average of 19% of the total L-ascorbic acid in the liver of the normal guinea pigs was found in the bound fraction compared to an average of 28% for the vit. C deficient animals (Table I). Sealock *et al.* (1,2) found that about 15% of the L-ascorbic acid in normal guinea pigs was in the bound fraction compared to 37% for vit. C deficient guinea pigs. Following the administration of D-ascorbic-1-C^{14} acid to vit. C deficient guinea pigs, about the same fraction of the total D-ascorbic acid in liver as in the case of L-ascorbic acid was found to be bound, averaging 35%.

Discussion. The results obtained in this study using C^{14} labeled L-ascorbic acid are in agreement with those determined colorimetrically by Sealock *et al.*(1,2) in that there is a bound form of L-ascorbic acid in guinea pig liver. At present no information is available on the nature of this binding. Sealock *et al.* (1,2) have attached importance to this bound form of L-ascorbic acid in the metabolism of tyrosine.

The results also show that D-ascorbic acid can exist in a bound form. In this connection it is of interest that D-ascorbic acid, which is reported to have no vit. C activity in guinea pigs(11), has recently been shown to be equally active as L-ascorbic acid in the *in vitro* metabolism of tyrosine(12).

Summary. Studies with C^{14} labeled L-ascorbic acid have yielded further evidence for the presence of a bound form of the vitamin in guinea pig liver. About 19% of the liver L-ascorbic acid is bound in normal and 28% in vit. C deficient animals. When D-ascorbic acid, the enantiomorph of L-ascorbic acid, is administered to vit. C-deficient guinea pigs, it was found in a bound form to about the same extent as L-ascorbic acid.

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Enhanced Absorption of Vitamin B_{12} in Gastrectomized Rat by Rat Intrinsic Factor.* (22253)

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The essential physiologic defect in Addisonian pernicious anemia and in the totally gastrectomized human subject is inability to assimilate vit. B_{12}. In both, this absorption defect can be diminished by the simultaneous administration of the so-called intrinsic factor of normal human gastric juice. This specific action of intrinsic factor is demonstrable following its simultaneous oral administration with vit. B_{12} in two ways: (a) hematopoietic effects in the anemic patient(1) or (b) enhanced uptake of radioactive vit. B_{12} in either the anemic or the non-anemic patient whether previously treated or not(2-4) including the gastrectomized patient(5). To date, no other method of detecting the activity of the intrinsic factor of normal human gastric juice has been found to be satisfactory.

The availability of radioactive vit. B_{12} labelled with Co^{60}(6), and especially of preparations of high specific radioactivity, suggested an attempt to develop an animal preparation for the detection of intrinsic factor. Although it was not known whether the rat, like man and the hog, secretes intrinsic factor, the availability of the species as a laboratory animal appeared to justify an effort to employ it. The fact that no increase, or indeed a decrease, in the uptake of radioactive vit. B_{12} had been found by others(7) to occur in the intact rat as a result of the simultaneous administration of intrinsic factor and vit. B_{12} was not discouraging, since this would be expected if the natural secretion of intrinsic factor was unimpaired and if non-specific binding(8) of Co^{60}-B_{12} by the intrinsic factor preparations had occurred. Our first interest was to discover whether the removal of the stomach and/or other portions of the gastrointestinal tract would reduce the ability of the rat to absorb radioactive vit. B_{12}. It was soon found, as recently reported by others(9), that with total gastrectomy this was so. However, it was considered that such a mutilating operation on the alimentary tract might well have had entirely non-specific effects on assimilation including vit. B_{12}. Consequently, only if an enhanced absorption of vit. B_{12} ensued in the operated animal when intrinsic factor was simultaneously administered by mouth could the conclusions be drawn with certainty that deficiency of intrinsic factor secretion (a) had been induced by the operative procedure and (b) had been temporarily abolished by the intrinsic factor administered. These conclusions appear to be justified by the present observations upon totally gastrectomized rats using Co^{60}-labelled radioactive vit. B_{12}, but only when normal rat stomach was the source of the intrinsic factor administered.

Methods. Male white rats of the Sprague-Dawley strain, weighing about 250 g were used. Total gastrectomy was done under Nembutal (pentobarbital sodium) anesthesia producing an end-to-end anastomosis of the lower end of the esophagus to the bisected pylorus. After brief experience the mortality at operation and during the following week was about 30%. Among animals surviving 2 weeks, only occasional animals lost weight and died. Gastrectomized rats were not used in experiments until at least 10 days after operation and only if they appeared to be in good health. At least 5 days elapsed between successive administrations of Co^{60}-B_{12} on the same gastrectomized animal. During the experiments the animals were kept in individual metabolism cages permitting total collection of stools without coprophagy. In order to

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minimize the bulk of the feces of which the radioactivity was to be measured, the diet supplied *ad libitum* consisted of a nutritionally complete synthetic low residue diet.[‡] It contained 18% casein as the source of protein, and among other essentials 1.35 mg vit. B_{12}/100 lb diet. On this diet the gastrectomized rats appeared to be healthy, gained weight and excreted normal appearing feces. After a preliminary fast of at least an hour each animal was lightly anesthetized with ether and given a single dose of Co^{60}-B_{12} followed immediately by the preparation to be tested for intrinsic factor activity. Both were in liquid form and were injected with different syringes in measured amounts by means of a small rubber catheter about 3 mm in diameter that had been cautiously inserted through the esophagus for a distance of 8-10 cm. The amount of radioactive vit. B_{12}[§] administered, which had a specific activity of about 1 μc/μg, was invariably 0.015 μg dissolved in 1 ml of water. The volume of the material to be tested for intrinsic factor activity was the same in each experimental group of rats and ranged in different experimental groups from 2-5 ml for a single dose. The stools were then collected over a 4-day period, digested with 20% NaOH and homogenized in a Waring Blendor. Thereafter 4 aliquot samples of 7.5 ml each were examined for radioactivity in a well-type scintillation counter and the individual values averaged. Human gastric juice was obtained from a normal fasting male following the injection of Histalog (3-beta aminoethylpyrazole dihydrochloride)(10). The juice was neutralized immediately after collection, passed through a coarse sintered glass filter and kept frozen until used. This procedure is known from many observations on patients with pernicious anemia(1) to provide an active source of intrinsic factor. As a source of hog intrinsic factor, a commercial preparation of hog stomach mucosa[‖] was used. As a potential source of "rat intrinsic factor" the entire stomachs of normal rats were homogenized in a Waring Blendor with water. For each stomach, which had been kept frozen since excision and which weighed about 1.5 g, 10 ml of water were used in preparing the homogenate which was filtered through gauze in order to remove coarse particles before use.

Rat gastric juice was obtained from normal animals fasted overnight following ligation of the pylorus. In the morning a variable degree of gastric distention was noted when the animal was killed and the gastric contents, averaging about 15 ml, were removed. The gastric juice so obtained was slightly turbid and sometimes brownish in appearance. The pH of a pooled sample from several rats was 1.3.

Results. The results of measurements of the radioactivity in the feces in single experiments upon 11 normal rats and in 61 experiments upon 14 gastrectomized rats are graphically presented in Fig. 1. Values are reported as percentage recoveries of radioactivity after single oral doses of Co^{60}-B_{12} together with water or with one of the preparations to be tested for intrinsic factor activity.

In *normal rats* a significant fraction of the test dose of Co^{60}-B_{12} in water was usually absorbed as inferred from the excretion of radioactivity. In the *gastrectomized rats* there was apparently no absorption of Co^{60}-B_{12}. When 2 ml of normal human gastric juice were used as a source of intrinsic factor, the absorption of vit. B_{12} did not improve, nor was the fecal excretion diminished when, instead of 2 ml of normal human gastric juice given once, 3 ml were given initially and were followed after an interval of an hour by 3 ml more. The administration of 4 mg and even of 20 mg of the preparation of hog stomach mucosa was also ineffective in decreasing the excretion of radioactivity.

On the other hand, when whole rat stomach homogenized in water was administered in a dosage of ½ of a stomach to gastrectomized rats the absorption of vit. B_{12} apparently became normal. When the dose was decreased to 1/10 of a stomach, the effect upon fecal

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[‡] Available commercially from Nutritional Biochemicals Corp., Cleveland, O.

[§] Kindly supplied by Dr. Nathaniel S. Ritter of Merck & Co., Rahway, N. J.

[‖] Obtained through the courtesy of Dr. Kenneth W. Thompson of Organon Inc., Orange, N. J.

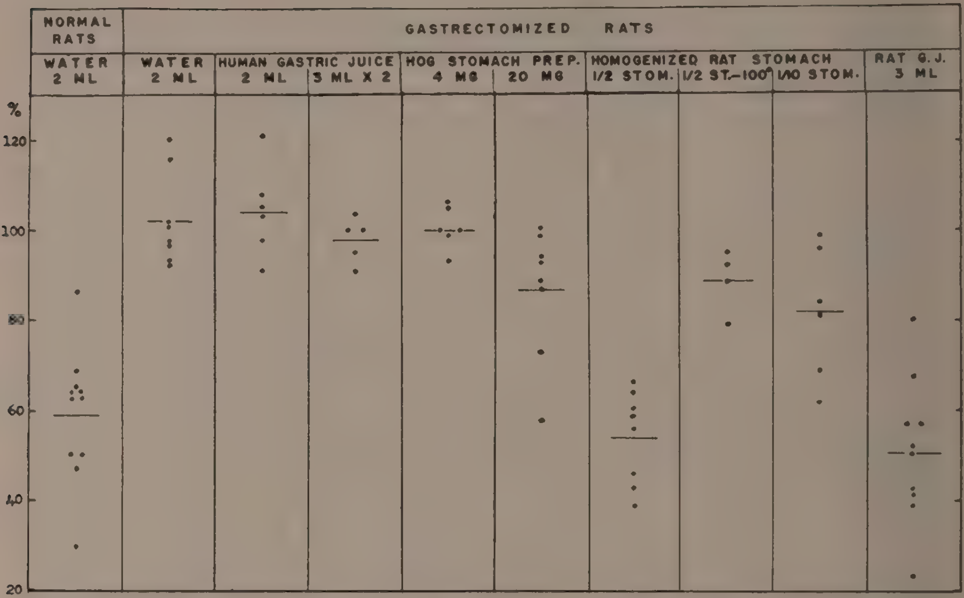

FIG. 1. Radioactivity recovered in the feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated above. Each dot represents the result of an experiment on a single animal; bars indicate averages.

excretion was at most slight. When 3 ml of rat gastric juice were administered, the average reduction in fecal radioactivity resembled that obtained with ½ of a homogenized rat stomach. Finally, when ½ of a homogenized rat stomach was administered subsequent to heating on a boiling water bath for 7 minutes, its effectiveness appeared to be greatly diminished.

Discussion. These results appear to demonstrate that total gastrectomy in the rat renders the animal unable to absorb a small test dose of radioactive vitamin B_{12} when given in an amount of which about half is absorbed by the normal animal. This disability is not associated with any gross signs of intestinal dysfunction, and may be largely abolished by the simultaneous administration of sufficient homogenized rat stomach or rat gastric juice. Taken together, these findings indicate that the assimilation of vit. B_{12} in the rat resembles that in man in that the stomach appears to be the exclusive or major site of secretion of a so-called intrinsic factor essential for that process. However, neither normal human gastric juice nor the preparation of hog stomach mucosa employed displayed with certainty any activity as intrinsic factor in the rat.

During the preparation of this manuscript our attention was drawn to the recent publication of Watson and Florey(11) demonstrating that after resection merely of the distal (secretory) portion of the rat stomach results were obtained that were generally similar to ours. Thus, their operated animals failed to absorb radioactive vit. B_{12}, and this difference from the intact animal was corrected by administration of extracts prepared from the distal portion of the rat stomach but not by administration of the secretion from a pyloric pouch created in the hog. It is a pleasure to report this confirmation of their observations and to point to their additional evidence that in the rat the secretion of intrinsic factor is largely by the distal portion of the stomach.[¶] Moreover, although not established by the experiments of Chow and his associates(9), it now appears probable that the defective absorption of Co^{60}-B_{12}

reported by them was in fact due to lack of rat intrinsic factor.

The apparent inactivity of normal human gastric juice and of hog stomach preparations employed by others(9,11) as well as here requires consideration. In our experiments both were obtained by methods repeatedly shown to conserve their intrinsic factor activity for patients with pernicious anemia. The doses of human gastric juice employed, 3 and 6 ml respectively, were for the rat relatively much larger than the 10 ml known to be hematopoietically active with as little as 1 μg of vit. B_{12} in pernicious anemia(12). The administration of 4 mg of the hog stomach preparation was without detectable effect as was also in all probability the administration of 20 mg. The larger amount constitutes about ½ of the amount of hog stomach extract present in 1 U.S.P. unit of a pharmaceutical preparation of vit. B_{12} with Intrinsic Factor Concentrate.** This is an effective daily oral dose in pernicious anemia when given with about 15 μg of vit. B_{12}. This hog stomach preparation contained about 80 $\mu\mu$g of normal vit. B_{12} in the 20 mg dose or far less than the 1680 $\mu\mu$g present in ½ of a homogenized rat stomach. Moreover, 6 ml of normal human gastric juice is said to contain only about 0.004 μg of vit. B_{12}(13). Thus, in these preparations insufficient normal vit. B_{12} was administered to compete significantly for absorption with the Co^{60}-B_{12} and so to account for their apparent inactivity in the rat. Although the inhibitory effect of non-specific binding(7,8) of the vit. B_{12} by the human and hog intrinsic factor preparations can not be excluded, the negative results with these preparations are consistent with the speculation of Chow and his associates (9) that intrinsic factor may possess species specificity. In this connection it is of interest to note that Toporek and his associates(14) found that, in contrast to the plateau effect of hog stomach preparations, progressively more Co^{60}-B_{12} was absorbed in pernicious anemia patients when normal human gastric juice was used as a source of intrinsic factor.

Conclusions. 1. The percentage excretion of radioactivity in the feces of normal and of totally gastrectomized rats, following administration of a single dose of 0.015 μg Co^{60}-B_{12}, was determined by scintillation counting. 2. The gastrectomized rats apparently were unable to absorb the labelled vit. B_{12} (100% fecal excretion). 3. Simultaneous administration of homogenized rat stomach or of rat gastric juice but *not* of human gastric juice or of an extract of hog stomach mucosa reproduced in the gastrectomized rat the capacity of the normal rat to absorb the test dose of Co^{60}-B_{12} administered (60% fecal excretion). 4. The rat, like man, secretes intrinsic factor largely, if not exclusively, into the stomach; but sources of human and hog intrinsic factor possess little or no activity in the rat. 5. The observations of Watson and Florey indicate that in the rat the secretion of intrinsic factor is probably confined to the distal secretory portion of the stomach.

The authors are grateful to Mrs. Rose B. Blumenthal and to Mrs. Ellen B. Block of the Thorndike Memorial Laboratory for technical assistance.

¶ Subsequently confirmed here in experiments upon 6 totally gastrectomized rats given ½ of the distal portion of a rat stomach after homogenization in water. The fecal excretions ranged from 54-72, average 60%, of the radioactivity administered. On the other hand, 5 similar experiments with the proximal portion of the rat stomach showed no effect upon fecal excretion: range 99-121, average 112%.

**Information kindly supplied by Dr. Kenneth W. Thompson of Organon Inc., Orange, N. J.

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Enzymatic Degradation of Side-Chain of Adrenocortical Steroids: Conversion of Hydrocortisone to Reichstein's E.* (22254)

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(Introduced by George Sayers.)

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In recent publications from this laboratory (1,2) an enzyme system found in both microsome and supernatant fractions of rat liver has been described which induces an alteration in the C-20 ketone group of adrenocortical steroids. Further observations on this system have been made and in addition positive evidence has been obtained that the reaction involving the C-20 ketone of the steroid molecule is reductive in nature.

Methods. Methods used in this study were identical with those described in previous publications(1,2).

Results. The first study in this series was undertaken to determine if the system reducing ring A could be separated from the system reducing the 20-ketone. A 0.25 *M* sucrose homogenate of rat liver free of nuclei and mitochondria was centrifuged at 20,000 X gravity for one hour. The supernatant fraction was discarded and the microsome fraction was resuspended and recentrifuged twice under the same conditions. Results obtained following incubation (37°C, 1 hr) of cortisone with the twice-washed microsome fraction are indicated in Fig. 1. It may be noted that the extent of Δ^4-3-ketone reduction in the complete system was only slightly less than that of 20-ketone reduction. Subsequent studies have confirmed this observation.[‡] These results are at variance with those of Tomkins and Isselbacher(3) who reported that the Δ^4-3 ketone reducing system is present only in the supernatant fraction of rat liver.

p-Chloromercuribenzoic acid (PCMBA) inhibition of the Δ^4-3 ketone reducing system and of the 20-ketone reducing system in the microsome fraction was investigated. Fig. 2

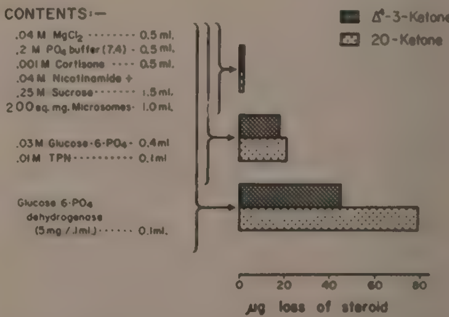

FIG. 1. Presence of both C-20 ketone and Δ^4-3-ketone reducing systems in microsome fraction of rat liver. See footnote (§) for definition of equivalent milligram (eq mg).

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[†] Data submitted in this report are in partial fulfillment for the Ph.D. degree. Predoctoral Fellow of the Arthritis and Rheumatism Foundation, 1955-1956. Present address: Upjohn Co., Kalamazoo, Mich.

[‡] Recknagel, R. O., unpublished.

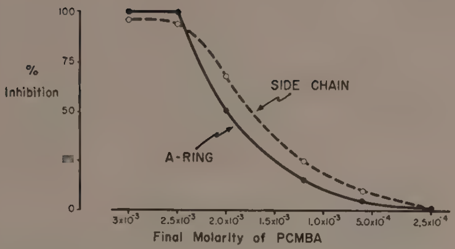

FIG. 2. Inhibition of Δ^4-3-ketone and C-20 ketone reducing systems by p-chloromercuribenzoate. Conditions as in Fig. 1.

indicates that inhibition of both systems was virtually complete at a final concentration of 3.0×10^{-3} *M* PCMBA. However, the inhibition by PCMBA appears to be due to inhibition of the TPNH generating system, since it was shown that glucose-6-phosphate dehydrogenase was strongly inhibited by PCMBA (Table I). The question of a dependence of C-20 and Δ^4-3-ketone reduction on SH-enzymes must therefore be considered as unsettled.

The enzymatic complexity of the microsome fraction with respect to reduction of steroid ketones is illustrated by the data of Fig. 3. In this study, equal quantities of cortisone, hydrocortisone, 11-desoxycortisone, tetrahydrocortisone, dehydroepiandrosterone, and androstenedione were incubated at 37°C for 2 hours using the conditions described in Fig. 1. Four-hundred equivalent milligrams§ of microsomes were used as source of enzyme. Loss of the C-20 group was followed by the Porter-Silber reaction(4). The Callow modification of the Zimmerman reaction(5) was used to follow disappearance of the 17-ketone group. The steroids employed have a variety of biological actions. Tetrahydrocortisone and 11-desoxycortisone are biologically inactive. It is apparent that the metabolism of these steroids is not related in a simple and obvious way to their metabolic effects. The metabolism of typical 17 ketosteroids (dehydroepiandrosterone, androstenedione) in the microsome fraction adds to the complexity. The fact that tetrahydrocortisone reacts to a greater extent than the other steroids tested was confirmed by additional investigations (Fig. 4). During the first 90 minutes of incubation the loss of the 20-ketone of tetrahydrocortisone was about twice the rate observed for hydrocortisone. These *in vitro* observations reflect the *in vivo* observations of others, in which it was shown that tetrahydrocortisone disappears from the blood faster than hydrocortisone when administered to experimental subjects(6). Also, studies of Sandberg *et al.*(7) indicate that cortisone disappears at a more rapid rate from the blood than hydrocortisone, and that the steroid degradation products of hydrocortisone appear in the urine at a less rapid rate than the degradation products of cortisone. Whether the metabolic rate studies recorded in Fig. 4 represent the enzymatic basis for the *in vivo* observations cannot be stated with certainty at this time.

TABLE I. Inhibition of Glucose-6-Phosphate Dehydrogenase by p-Chloromercuribenzoate.

	E_{310}*
Complete system	.465
" " $+ 1 \times 10^{-3}$ *M* PCMBA	.061
" " + 2 " "	.011
" " + 3 " "	.035

* Optical density reading at 340 mμ one min. after start of reaction.

Conditions: Sigma Chemical Co. Bulletin No. 201, Nov. 1951.

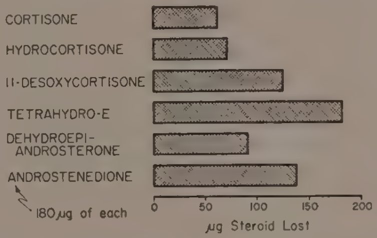

FIG. 3. Enzymatic complexity of rat liver microsomes with respect to reduction of steroid ketone groups.

Isolation of Reichstein's E. Seven and two-tenths milligrams of hydrocortisone (free alcohol), dissolved in 20 ml of 0.25 *M* sucrose made to 2% with ethanol were incubated with 20 equivalent grams of rat liver microsomes (washed 2 times with 0.25 *M* sucrose). The

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§ An equivalent mg of microsomes represents all of the microsome fraction obtained under prescribed centrifugation conditions from one mg wet weight of whole liver.

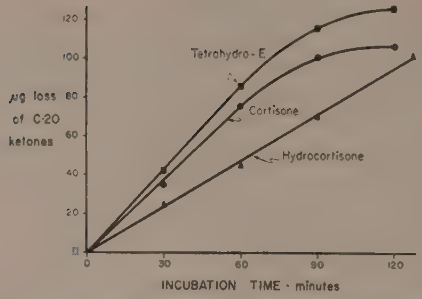

FIG. 4. Relative rates of reactions of various steroids in rat liver microsome fraction. Conditions as in Fig. 1.

following cofactors were added to the incubation medium: 14.0 ml of 0.03 *M* glucose-6-phosphate, 20.0 ml of 0.02 *M* phosphate buffer (pH 7.4), 10.0 ml of 0.04 *M* $MgCl_2$, 10.0 ml of 0.04 *M* isocitrate, 10.0 ml of 0.01 *M* TPN. The pH of the medium (7.4) was checked before incubation and 10.0 mg of glucose-6-phosphate dehydrogenase were added immediately before evacuation. The enzyme mixture was transferred to a 37°C water bath and incubated for 45 minutes. The aqueous phase was extracted 3 times with 400 ml $CHCl_3$. The $CHCl_3$ extract was dried under a stream of air and the residue redissolved in 70% ethanol and subsequently partitioned between 70% ethanol and pentane for removal of interfering substances. The pentane fraction was discarded and the 70% ethanol phase dried under vacuum. The dry residue was taken up in a small amount of chloroform and placed on a Florisil column (8). The column was eluted with 100 ml of chloroform, 100 ml of 2% methanol:chloroform, and 200 ml of 25% methanol:chloroform. The eluates were dried and the residues dissolved in methanol. One-twentieth of each eluate was applied to a paper strip and chromatographed in the benzene: 50% methanol:water system of Bush(9). Paper chromatograms were observed under ultraviolet light and subsequently sprayed with 4% ethanolic phosphomolybdic acid. Observation under the ultraviolet lamp revealed the presence in the 25% methanol-chloroform eluate of a compound more polar than hydrocortisone. The other eluates contained no detectable steroids. The phosphomolybdic acid reagent revealed (blue spot) the presence in this same eluate of a still more polar compound which was not visible under the ultraviolet lamp. In order to remove unreacted hydrocortisone the remainder of the 25% methanol-chloroform eluate was dried under vacuum and the dry residue placed on eight 1½-inch wide paper strips and chromatographed as before. The paper chromatograms were observed under the ultraviolet light. Regions which corresponded to hydrocortisone were discarded. More polar regions were eluted with methanol, dried under vacuum, redissolved, and a sample which corresponded to

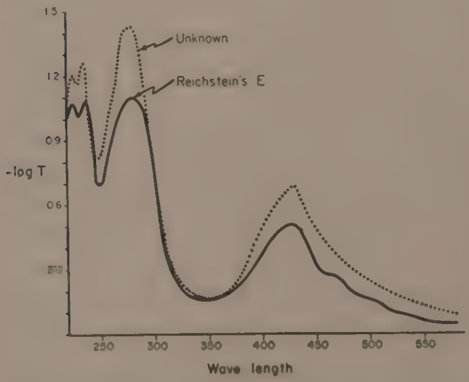

FIG. 5. Sulfuric acid induced absorption of unknown steroid compared to authentic sample of Reichstein's E.

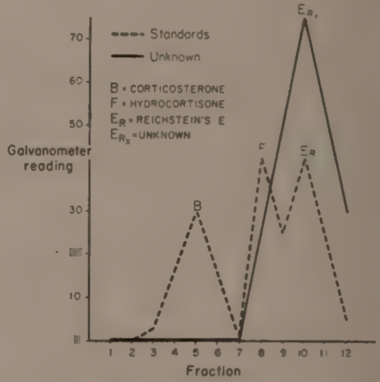

FIG. 6. Silica gel chromatography and fluorescence of unknown steroid compared to authentic sample of Reichstein's E.

one-twentieth of the original was rechromatographed in benzene:25% ethyl acetate. Ultraviolet and phosphomolybdic acid analysis revealed the presence of the 2 polar steroids as expected. In order to separate the 2 unknown steroids, the remainder of the methanol eluate was dried and rechromatographed on six 1½-inch wide paper strips in benzene:25% ethyl acetate. The unknown steroid which was not visible under the U-V lamp was not further analyzed. The areas on the 6 strips which were visible under the U-V lamp were eluted with methanol. In order to further purify the unknown steroid, the combined methanol eluates were rechromatographed on four 1½-inch wide paper strips in the benzene:25% ethyl acetate system. The ultraviolet absorbing steroid was eluted from the several strips, combined, dried under vacuum and diluted to 20.0 ml with absolute methanol.

The following tests with the recorded results indicate that the unknown compound was Δ^4-pregnene-11 β, 17 α, 20,21-tetrol-3-one (Reichstein's substance E). The stereochemical configuration of the C-20 alcohol of the experimentally derived product has not been definitively established.

Tests	Results
1. Rf values of unknown and known (free alcohols), on Bush(9) and Zaffaroni(10) systems.	1. Same Rf value for unknown and authentic sample of Reichstein's E.
2. Ultraviolet absorption spectrum at 220 to 260 mμ.	2. Absorption maximum at 240 mμ = Δ^4-3-ketone.
3. Phenylhydrazine reaction of Porter-Silber (4).	3. Unknown did not peak at 410 mμ. Not a C-20 ketone.
4. Sulfuric acid chromogen spectrum.	4. Spectrum (330 to 600 mμ). Identical with Reichstein's E. See Fig. 5.
5. Unknown chromatographed on silica gel column according to the method of Sweat(11).	5. Fluorescence and chromatographic behavior identical with Reichstein's E. See Fig. 6.
6. Unknown acetylated with pyridine-acetic anhydride 16 hr at room temp. Acetates of unknown and Reichstein's E chromatographed on Bush(9) and Zaffaroni(10) systems.	6. Rf values same for single chromatograms as well as mixed acetates.

Quantitative analysis at 240 mμ and calculation from known standards indicated that 2.1 mg of Reichstein's E were obtained from 7.2 mg of hydrocortisone. The more polar compound has not been definitely identified, but the evidence obtained suggests that it is a pentol. That the reactions in this system are probably exclusively reductive in nature was shown when 4-C^{14} hydrocortisone‖ was incubated with the preparation described, and the extracts chromatographed on the Zaffaroni system(10) for 92 hours. No C^{14} was present in the run-off from the paper strips. If oxidative reactions had occurred, 17-ketosteroids labelled with C^{14} would have been expected to appear in this fraction.

Conclusions. 1. Observations concerning the enzyme systems of the microsome fraction of rat liver which effect the C-20 ketone reduction of adrenocortical steroids have been described. 2. One product of hydrocortisone conversion in the microsome fraction of rat liver has been identified as Reichstein's E. The presence of at least one other product was noted.

We are indebted to Dr. John Schneider of the Jefferson Medical School for his generous gift of Reichstein's E, and to Dr. Constance de Courcy of the Jefferson Medical School for introducing us to the technics of the Bush system of paper chromatography.

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Steroid-Induced Adrenal-Pituitary Hypofunction II. (22255)

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It has been demonstrated that daily administration of hydrocortisone to rats lowers their tolerance to intraperitoneal injection of potassium chloride(1). Since this observation suggested adrenal-pituitary hypofunction, efforts were made to determine if these animals could be protected by treatment with various hormones. Intravenous administration of ACTH or aldosterone immediately prior to the injection of potassium chloride increased the tolerance of steroid-treated animals to KCl stress(2). It was observed that the same response was obtained if animals were given 1% NaCl to drink for 18 hours prior to injection of potassium chloride(3).

The protective action of sodium chloride suggested that the mortality of non-protected animals was not due to a hypofunctional adrenal or pituitary but rather to an electrolyte disturbance as a result of steroid treatment. The present experiments were designed (1) to measure the amount of sodium and potassium excreted in the urine during steroid administration, and (2) to determine the tolerance to potassium stress in rats depleted of sodium and potassium.

Methods. 1. Male rats, averaging 160 g body weight were placed in metabolism cages (2/cage) and fed Ingle's liquid medium carbohydrate diet *ad libitum*(4). Following a 2-week control period, these animals were injected once daily for 10 days with either 16% gelatin, 4 mg, hydrocortisone (F) in 16% gelatin, or 6 mg 11β, 17α-dihydroxy-4-pregnene-3,20-dione (21-desoxy-F) in CMC.* Urine volumes were recorded daily and urinary sodium and potassium were determined in a flame photometer. 2. Male rats were given intraperitoneal injections of 8 mg Thiomerin sodium (Wyeth), 5 mg Neohydrin (Lakeside), or 6 mg Diamox (American Cyanamid) in 0.2 cc aqueous solution. The rats were placed in metabolism cages, fasted for 18 hours and then injected with 0.16 M potassium chloride (5 cc/100 g body weight). The 18-hour urine collections were analyzed for sodium and potassium.

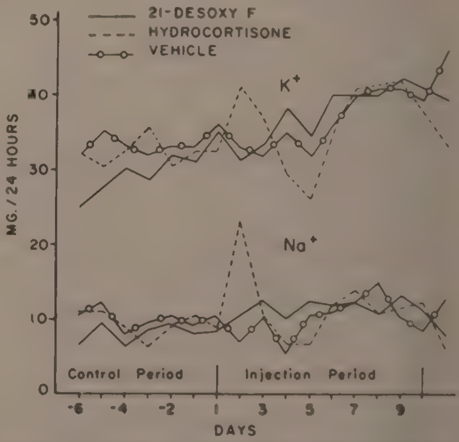

FIG. 1. Effects of hydrocortisone and 21-desoxy F on urinary sodium and potassium excretion.

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* 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol and 0.9% sodium chloride.

TABLE I. Effects of Various Diuretics on Urinary Excretion of Sodium and Potassium.

No. rats	Treatment	% survivals*		Sodium, mg/18 hr	Potassium, mg/18 hr
49†	4 mg F/day/10 days	19	—	—	—
15	Controls	73	S‡ 11	10.4 ± 4.73	33.0 ± 3.20
			D 4	8.62± 3.87	34.6 ± 4.13
15	Thiomerin, 8 mg I.P.	73	S 11	13.5 ± 3.87	37.7 ± 7.01
			D 4	10.37± 2.80	35.75± 1.69
15	Neohydrin, 5 mg I.P.	53	S 8	14.3 ± 4.85	40.7 ± 9.03
			D 7	15.5 ± 7.09	43.8 ± 9.56
15	Diamox, 6 mg I.P.	66	S 10	23.8 ± 6.13	43.9 ± 8.73
			D 5	24.6 ± 6.63	42.0 ± 9.54

* Stressed with 0.16 M KCl, 5 cc/100 g body wt. † Collins(1). ‡ S = Survivals; D = Deaths.

Results. Fig. 1 demonstrates the pattern of sodium and potassium excretion during daily administration of F or 21-desoxy-F for 10 days. Urinary excretion of sodium and potassium increased during the 24-hour period following the injection of F. This increased excretion was not maintained. The animals showed a decrease in the excretion of both sodium and potassium during the third day of treatment. Decreased urinary excretion of sodium and potassium coincided with a period of decreased food intake. By the sixth day, food intake was again normal and urinary electrolyte concentrations paralleled that of control animals.

Animals injected with 21-desoxy-F demonstrated a more gradual and sustained increase in sodium and potassium excretion. However, the increased urinary concentration of these ions was not too different from the values observed in control animals.

Table I gives the effects of various diuretics on the urinary excretion of sodium and potassium as well as the results following potassium stress. Urinary excretion of sodium and potassium in the Diamox-treated group is significantly higher than the control group. However, within any given group, there is no significant difference between the animals that survived the stress and those rats in which the potassium chloride proved fatal. Fifty-three per cent of the Neohydrin-treated animals survived after potassium stress. However, this survival rate is higher than the survival rate for hydrocortisone-treated animals.

Discussion. Dietary potassium enters the blood stream and becomes available to the cells via the extracellular fluid. When potassium is presented to the cells, the amount taken up is dependent upon cellular requirements. If the supply exceeds cellular demands, the excess potassium is presumably eliminated by renal excretory mechanisms. Increased concentrations of extracellular sodium dilutes extracellular potassium and increases renal elimination of potassium which results in a loss of intracellular potassium. These actions of sodium make it useful in the treatment of potassium intoxication. Conversely, depletion of sodium enhances the possibility of potassium intoxication(5-7).

Although the mechanism of potassium stress in animals with hypofunctional adrenal cortices has not been defined, a hydrocortisone-induced increase of sodium excretion is suggestive of a decreased tolerance to a potassium load. However, the present data indicate that marked sodium depletion does not decrease the resistance of rats to potassium stress if adrenal-pituitary function is not impaired. The sodium losing effect of hydrocortisone is of 1-3 days duration and is not sustained during chronic administration. This is in agreement with the observations of Ingle *et al.*(8). The data which have been presented above indicate that decreased resistance to a potassium load results from a decrease in adrenal-pituitary function and not from steroid-induced changes in electrolyte composition of the organism.

Summary. Data are presented which indicate that marked sodium depletion does not alter the resistance of intact rats to a potassium load. Early but transitory urinary electrolyte changes were observed during chronic hydrocortisone treatment. The decreased resistance to potassium stress in animals treated with hydrocortisone is probably due to hypofunction of the adrenal-pituitary axis rather than to steroid-induced alterations of electrolyte balance.

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Effect of Choline, Heparin and Aureomycin on Fatty Livers of Dogs.* (22256)

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Gyorgy, *et al.*(1,2) demonstrated that Aureomycin exerts a lipotropic and anti-cirrhotic effect in rats fed a high fat, low protein, choline deficient diet. Baxter and Campbell (3) noted prevention of severe renal injury and some diminution of liver fat after Aureomycin therapy. More recently the amount of fat in livers has been reduced in cholesterol-fed rabbits(4) and rats(5) by the daily administration of heparin. It therefore became of interest to compare the effectiveness of choline, Aureomycin and heparin in a different species, the dog, and to ascertain whether the mechanism of lipotropic action of Aureomycin was related to the choline sparing effect of this drug(3,6).

Methods. Healthy mongrel dogs, previously maintained on Purina laboratory chow were fed 14 g per kg body weight of a high-fat, low-protein, choline-deficient diet‡(7) for 21 days. Eight of these animals received 0.5 g of Aureomycin§ (Chlortetracycline) in capsule form in the morning and late afternoon simultaneously with the feeding of the diet. Seven animals received daily oral supplements of 1% choline chloride.‖ Nine other animals were injected twice a day with heparin§ 2 mg/kg body weight intramuscularly. On the 21st day, some animals were killed by rapid intracardiac air injections and liver slices were made by a Martin slicer(8). Liver slices were incubated with radioactive phosphate for one hour, with and without the *in vitro* addition of choline chloride (1 mg/cc of medium). Composition of medium and isolation of phospholipides have been described(6). From the specific activity of the inorganic phosphate of the medium and the total radioactivity in the phospholipide, the *in vitro* rate of phosphatide synthesis was calculated. The remaining dogs were sacrificed after the 3 week experimental period. Phospholipide phosphorus was determined on an

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† This study was carried out during tenure of Public Health Service Predoctoral Research Fellowship of National Institutes of Health, Public Health Service.

‡ The diet was composed of 38% lard, 44% sucrose, 8% vitamin test casein, 3% cod liver oil and 5% Cowgill's salt mixture. Any animal that did not consume entire diet upon feeding was force-fed.

§ Kindly donated by Lederle Co., Pearl River, N. Y.

‖ Kindly donated by Merck and Co.

TABLE I. Effect of Choline, Heparin and Aureomycin on Liver Lipide Partition.*

Group	No. animals	Phosphatide	Total cholesterol	Total lipide	Triglyceride + cholesterol ester fatty acid
Normal	7	29.4 ± 1.9	2.43 ± .13	43.1 ± 2.1	12.4 ± 1.7
HF†	11	20.1 ± 1.2	2.68 ± .14	64.7 ± 5.2	42.0 ± 4.0
" + choline	7	21.5 ± 1.6	2.32 ± .22	35.9 ± 2.4	12.2 ± 1.1
" + aureomycin	8	17.1 ± 1.2	2.92 ± .22	94.5 ± 14.1	73.3 ± 13.9
" + heparin	9	16.9 ± 1.1	2.46 ± .30	47.6 ± 3.5	28.3 ± 4.1

* Lipide values expressed as mg/g fresh tissue wt (mean ± stand. error).
† HF = High fat.

alcohol-ether, petroleum ether extract of liver (9). Total cholesterol was analyzed by the method of Sperry and Webb(10) and total lipides were gravimetrically determined by evaporating an aliquot of the petroleum ether extract. The difference between the total lipide on the one hand and the amount of phospholipide (lipide P x 25) plus cholesterol on the other was designated as triglyceride plus cholesterol-ester fatty acid fraction.

Results. The lipide partition in the livers of dogs on the various regimens is presented in Table I. The liver phosphatide concentration of animals on laboratory chow was significantly greater than that in any of the other groups. Neither treatment with heparin or Aureomycin, nor addition of daily adequate choline supplements had any effect on the depressed phosphatide levels of dogs maintained on the high-fat diets. Thus it appears that the decrease in liver phosphatides of dogs on the basic high-fat, low-protein diet was not the result of a simple choline deficiency but may be related to absence of adequate amino acids or other factors from the diet.

In contrast to the decrease in liver phosphatides, the cholesterol concentration was remarkably constant in all animals. The greatest alterations occurred in the "triglyceride" fraction (last column of Table I). Since the total cholesterol did not differ greatly from one group to another and constituted only a small fraction of total liver lipides, it is reasonable to assume that the different values in the last column of Table I primarily reflect alterations in triglyceride concentrations. Thus we may conclude that supplementing the high fat diet with 1% choline during the 3 week experimental period maintained triglycerides at normal concentrations. Administration of heparin to dogs on the high-fat diet appeared to exert some lipotropic effect since lowering of liver triglycerides was statistically significant at the 3% level of confidence. Aureomycin, on the other hand, did not exert any lipotropic action. Quite to the contrary, it appeared that triglyceride levels in Aureomycin treated animals exceeded those of the untreated controls on the same diet (P = 2.5%).

From these experiments one might conclude that Aureomycin aggravated the degree of choline deficiency, but the next set of experiments throws additional light on these results. Table II summarizes data on the rate of phosphatide synthesis by liver slices incubated with radioactive phosphate in the presence and absence of choline in the medium. The first 3 rows confirm our earlier findings (6) that *in vitro* addition of choline markedly stimulates formation of P^{32} labeled phospholipides in choline-deficient liver slices, but has practically no effect on liver slices from dogs maintained on Purina laboratory chow or an high-fat, low protein diet supplemented with 1% choline. If the high triglyceride content of livers of Aureomycin-treated dogs was the result of a choline deficiency similar to that of the animals on unsupplemented high-fat diet (second row of Table II) one would expect a marked increase in phosphatide synthesis of slices in choline supplemented baths. That this is not the case may easily be seen from the figures in the bottom row of Table II. Slices incubated in choline supplemented medium showed a slightly greater synthesis of radioactive phospholipides than slices incubated without choline, but the difference did not compare to the

TABLE II. Phospholipide Concentrations* and Rates of Synthesis in Liver Slices† with and without Added Choline.

Group	mg phospholipide P/g		γ P converted to phospholipide/hr/g	
	Without choline	Choline added	Without choline	Choline added
Normal	.93 ± .052	.99 ± .044	11.8 ± 2.5	12.9 ± 1.2
HF‡	.60 ± .049	.68 ± .063	9.5 ± .7	21.9 ± 2.3
" + choline	.73 ± .060	.75 ± .056	8.6 ± .3	9.7 ± .9
" + aureomycin	.57 ± .073	.58 ± .053	10.8 ± 2.0	14.2 ± 2.1

* Concentrations expressed as mg of phospholipide phosphorus/g fresh liver. Values are given as mean of 4 dogs ± stand. error.

† Each animal liver furnished 6 slices; 3 incubated with choline, 3 without; one slice/flask; each slice was analyzed separately.

‡ HF = High fat.

nearly 2½ fold increase observed in animals with known choline deficiency. According to this test, therefore, Aureomycin decreased the severity of choline deficiency. The inability of Aureomycin to influence the rate of phospholipide synthesis of liver slices agrees with the report of Cornatzer and Gallo(11) that this antibiotic had no effect on phospholipide turnover in the liver of intact rats.

Discussion. It might be well to emphasize that our results were obtained on adult dogs maintained on a high fat, high carbohydrate diet, deficient in choline and methionine, and possibly also in other unknown factors. The fact that addition of choline to our basal ration completely restored the normal appearance and normal triglyceride content of livers should not obscure the fact that the choline supplement failed to restore normal liver phospholipide levels. As a matter of fact, this finding constitutes perhaps one of the most interesting aspects of our study, namely, that the frequently observed parallelism between the lipotropic effect of choline and its effect on liver phospholipide metabolism may not be a cause and effect relationship, but may be more of a coincidental phenomenon. In the present study, for example, the *daily additions* of choline to high-fat diet exerted a marked lipotropic effect but failed to raise the liver phospholipide concentration or the rate of phospholipide synthesis in the liver slice (8.6 γ/g/hr as compared to 9.5 γ/g/hr). This finding is difficult to reconcile with the well known phospholipide-turnover-stimulating action of a single dose of choline given to choline-deficient intact animals(12), or added to their liver slices(6,12).

Gyorgy(2) employing a diet similar to ours observed that in rats Aureomycin markedly decreased liver lipide concentrations. Kaplan *et al.*(13) employing blood lipide alterations as his criterion concluded that in pancreatic duct-ligated dogs, Aureomycin exerted some lipotropic activity. Some choline sparing action appeared to occur in our animals as demonstrated by the failure of the liver slice to respond to added choline. However, this choline made available by Aureomycin was not effective in lowering liver lipides. Since large doses of Aureomycin have been reported to cause fatty livers in mice(14), we fed 1 g of Aureomycin per day for 3 weeks to 2 dogs maintained on Purina laboratory chow without any visible or chemical effects on liver lipides. Similar results were reported by Kaplan *et al.*(13) and Sutherland *et al.*(15). Thus, administration of this amount of Aureomycin to dogs on a nutritionally adequate diet was not associated with fatty liver development. Under the conditions of our experiments Aureomycin apparently exerted some choline sparing action without a concomitant lipotropic effect on the liver, but such an hypothesis deserves further studies.

Summary. The triglyceride concentration in livers of dogs maintained for 3 weeks on a high-fat, low-protein, choline-deficient diet was lowered by daily injections of heparin and by supplementation of the diet with choline. Oral administration of Aureomycin increased liver triglyceride concentrations. Choline, heparin or Aureomycin did not alter liver phosphatide or cholesterol concentra-

tions of animals receiving the high-fat diet. Aureomycin, *per os*, did not affect *in vitro* synthesis of liver phosphatides, but modified the response of liver slices to the *in vitro* addition of choline.

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Rapid Procedure for Erythrocyte Packed Cell Volume and Sedimentation Rate Determinations. (22257)

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During laboratory investigations involving large numbers of human blood samples, erythrocyte sedimentation rates and packed red cell hematocrit values were required for correlation with other observations. This need stimulated efforts to develop time saving modifications of the conventional procedures primarily to devise a rapid preliminary screening operation for selection of abnormal samples for further study by standard methods. However, in actual practice the rapid procedure yielded accuracies sufficiently comparable to standard methods that its routine substitution for the more time-consuming technics appeared possible. The procedure was put on a quantitative basis and appropriate comparison studies with accepted methods(1,2) were carried out. Results here presented indicate that the values are satisfactory for routine clinical use and that they may be obtained with substantial time saving and other economies. The method has been used in testing over 10,000 patient blood samples. The essence of the modification consists in determining sedimentation rate and hematocrit values directly in the original blood collection tube without necessity of transfer to a secondary, calibrated hematocrit tube. The recent introduction of rubber stoppered, evacuated, blood collection tubes containing appropriate anti-coagulants contributes to the speed and convenience of this technic. However, any standard test-tube or collection tube may be substituted or adapted to this procedure. A basic problem of this modification, the variable amount of blood sample in each collection tube, has been overcome by use of a proportional volume chart accurately indicating percentage of relative parts of a test tube blood sample irrespective of its total volume (Figs. 1 and 2). The

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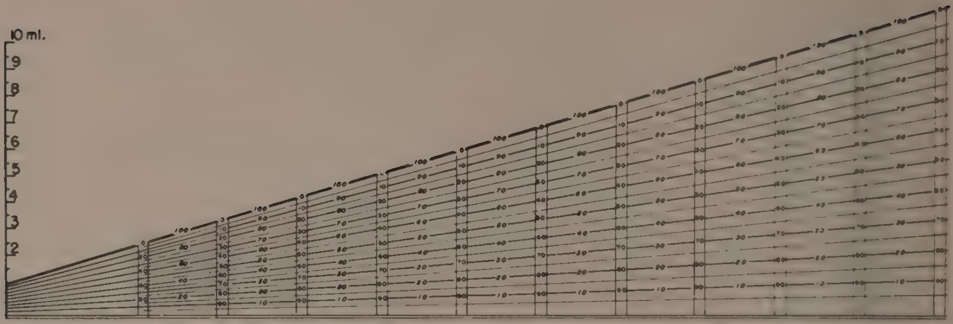

FIG. 1. Proportional volume chart for determining relative percentage of separated substances in an uncalibrated tube irrespective of volume fluctuations.

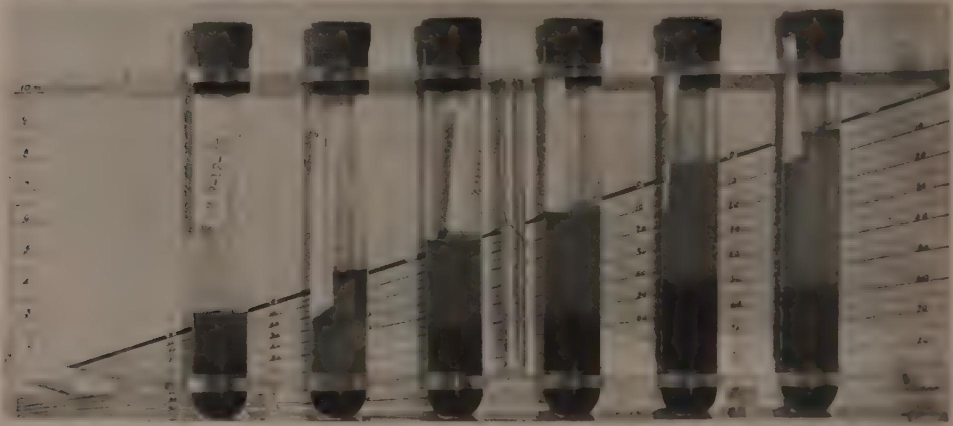

FIG. 2. Blood collection tubes arranged to illustrate use of chart in determining sedimentation rates or hematocrit values irrespective of sample volumes.

design of the chart takes into consideration corrections necessary for the spherical bottom of the tube, the diameter, shape and thickness of glass, and the meniscus. The chart (Fig. 1) was designed for standard blood collection tubes[†] used in our studies but, as can be readily shown, a reasonable degree of variation in these factors can be tolerated.

Method. Erythrocyte sedimentation rate. Blood is collected by standard venepuncture procedure, employing a syringe and test tube with suitable anticoagulant, or the commercially available rubber-stoppered Vacutainer[†] tube used with double-ended needle and holder. These studies employed the evacuated tubes containing either heparin or oxalate. The sealed samples are numbered, or otherwise identified, and placed in racks suitable for holding the tubes perfectly perpendicular (Fig. 2).[‡] An optional procedure found useful in sharpening the plasma meniscus is the addition of silicone anti-foam emulsion.[§] A tiny drop is added to each tube with a No. 24 needle and a one-ml tuberculin syringe. The blood is mixed thoroughly by inverting the entire rack vigorously 10 times, and is then placed on a level vibrationless surface to sediment for 30 minutes. If more than one rack is run simultaneously, the mixing interval between racks should correspond to the rack reading time of the technician. This interval is approximately one minute with a 10-tube rack for an experienced operator. If a time course curve is desired, appropriate interval readings may be taken, provided tube contents are not unduly disturbed. At termination of the sedimentation period, relative rates are read by placing the rack of tubes in front of the reading chart and sliding the entire rack to the left or right as required to cause the top of the blood sample of a given tube to coincide with the upper heavy meniscus line of the chart. The sedimentation rate of the red cells is then read from the appropriate chart line by noting the position on the chart of the erythrocyte-plasma interface. Either edge of tube may be employed in making the reading but proper eye level should be carefully observed to avoid parallax errors. It will be noted that the chart has 2 reading scales. The numbers reading from top down are for red-cell sedimentation rate, while those reading from bottom up refer to packed red cell volume. If repeat determinations are desired, the entire rack or a given sample may be remixed and reread as many times as required, the only limitation being possible changes in sedimentation rate with time. *Packed red cell volume.* The hematocrit value, or red cell volume, is obtained by transferring the blood collection tubes to a suitable centrifuge and spinning for 10 minutes at 2000 rpm (mean RCF = approximately 700). The large, 4-place horizontal head, No. 284 or 289, of the standard International centrifuge is convenient since each cup will handle from 8 to 12 tubes depending upon diameter of tube used, permitting a total load of 32 to 48 blood samples. Care should be taken in packing the tubes that they are as nearly vertical as possible so that the packed red cell interface will be level in the tube for optimum accuracy in reading. At completion of the run, hematocrit values are read in the same manner as sedimentation rates except that numbers running from bottom of chart upward apply. The percentage of white cells and platelets, or buffy coat, are noted in the usual fashion and their percentage may be read from the chart. Icterus or other abnormalities of the plasma may be recorded as hematocrits are read.

[†] The Becton, Dickinson, and Company Vacutainer blood collection tubes containing laboratory grade heparin or mixture of dry ammonium and potassium oxalate.

[‡] Chart, racks, and accessory equipment manufactured by Virtis Co., Yonkers, N. Y.

[§] Dow Corning antifoam A emulsion, a silicone defoamer.

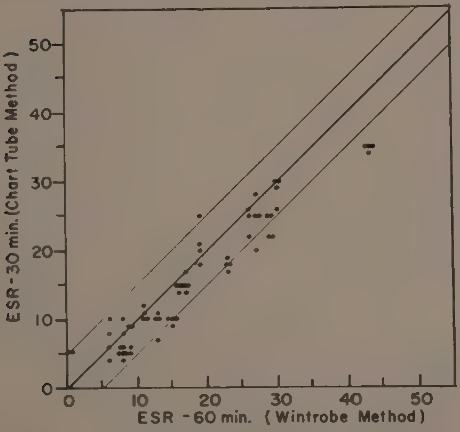

FIG. 3. Values obtained throughout the erythrocyte sedimentation rate range with tube-chart procedure as compared with Wintrobe technic. (Tube values are plotted about the straight line representing Wintrobe values. Scattering reflects combined errors of both methods. Boundary lines equal plus or minus 5 reading points.)

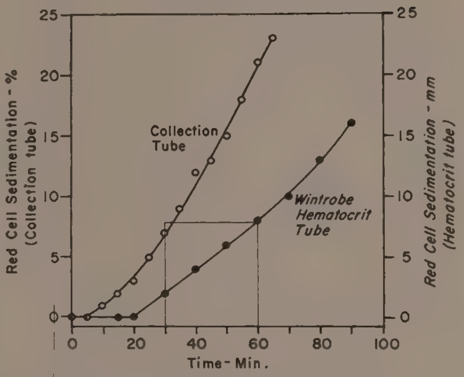

FIG. 4. Comparison of time-course curves of same blood sample determined by tube-chart and Wintrobe procedures.

Results. Erythrocyte sedimentation rate. A comparison of results between this procedure and the conventional Wintrobe method is illustrated in Fig. 3 and 4. In Fig. 3, values for a group of blood samples covering a wide range of sedimentation rates are plotted to illustrate normal scattering of points obtained by this method at all levels of sedimentation rate when referred to Wintrobe values. This was done by employing a standard time factor of 30 minutes in the case of the tube-chart method as compared with 60 minutes with the Wintrobe hematocrit tube. It may be seen that over 80% of the points fall within the plus or minus 5% boundaries established by the standard Wintrobe procedure on the same blood samples. It will be remembered that in this type of graphic plot the combined errors of both methods are reflected in the scattered points about the line. The aberrant points thus may be due to an excessive error by either method or to the smaller combined errors in the same direction by both methods.

Fig. 4 shows the time course plot of the same blood sample when run by the 2 methods. Differences in slope of rate curves illustrate the basis for approximate equivalence of 30- and 60-minute readings for the 2 methods. It is seen that the vertical lines at these 2 time periods intersect the sedimentation curves at approximately the same reading level. This slope increase has the advantage of shortening the time requirement for sedimentation to reach a desirable measuring point, and the disadvantage of increasing the critical importance of the time factor. Thus a lag of 2 minutes in reading the ESR by the tube-chart method would cause a greater error than the same lag period with the Wintrobe hematocrit. Although 30-minute and 60-minute readings are not always equivalent they are statistically close enough to take advantage of the convenience of the 30-minute time period.

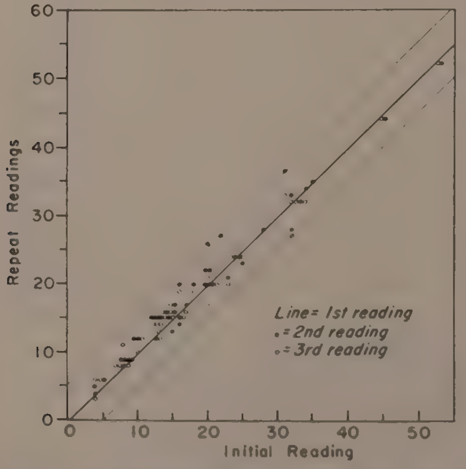

FIG. 5. Reproducibility of repeated ESR determinations over a wide range employing tube-chart technic.

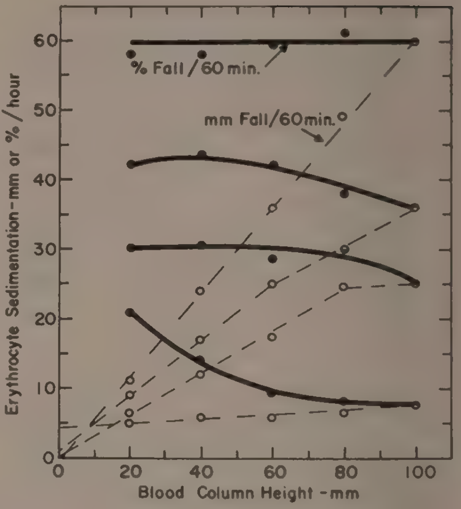

FIG. 6. Comparison of mm and percentage sedimentation rate readings employing identical blood samples with different column heights. Four levels of sedimentation rate are illustrated. Solid lines = percentage sedimentation rate (% of total height /60 min). Broken lines = mm of red cell sedimentation/60 min.

Reproducibility of this procedure with repeat determinations on the same blood samples over a wide ESR range is illustrated in Fig. 5. The original readings are plotted as the diagonal reference line and subsequent second and third readings are plotted as the closed and open circles. The moderate degree of scattering throughout the entire range indicates that reproducibility is not biased by magnitude of the sedimentation rate.

The published procedures for measuring erythrocyte sedimentation rate employ "millimeters" of fall of the top boundary of the settling red cell population. The several technics in current clinical use employ hematocrit tubes of varying lengths fluctuating from 40 to 200 mm(2-5). Since the procedure reported here employs "percentage" of fall rather than absolute distance in mm it was necessary to investigate the relationship between absolute and percentage measurements within the anticipated extremes of sample volume fluctuation and thus of blood column height. Under conditions of our testing operations the extremes of whole blood sample volume gave column heights of 20 to 80 mm with the preponderance of samples from 50 to 70 mm in height. Fig. 6 illustrates the relationship between absolute mm of fall and percentage, or relative fall, when testing identical blood samples of different height. The data also show the relationship between blood samples with widely differing sedimentation rates.

For all sedimentation rate levels except the lowest one, the blood column height has little influence on percentage fall, whereas there are substantial differences in the reading of various height tubes when mm are the criteria of measurement. This accounts for some of the numerical confusion in attempting to establish normal and abnormal standards of sedimentation rate between several methods utilizing different column heights(2-7). With the percentage technic described here the sample column height is not critical between the range of 20 to 100 mm unless the ESR is low, in which case samples less than 40 mm in height should be avoided, corrected, or transferred to a smaller bore tube which will increase the sample height to 50 or 60 mm.

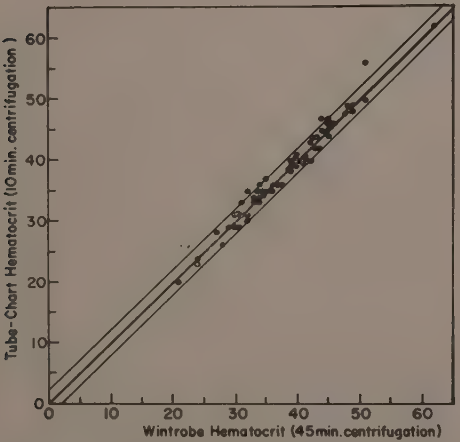

FIG. 7. Comparison of hematocrit values on 68 blood samples determined by both tube-chart and Wintrobe procedure employing 10 and 45 min. centrifugation respectively. (ref = 700, rpm = 2,000.)

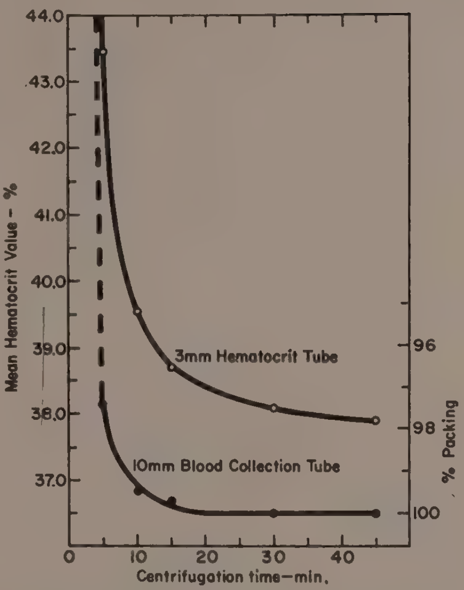

FIG. 8. Comparison of sedimentation curves in centrifugal packing of red cells when employing a 13 mm blood collection tube and a 3 mm hematocrit tube. These curves are mean values of 20 samples run simultaneously from the same group of standard hospital blood samples.

The foregoing data illustrate that the expression of erythrocyte sedimentation by percentage fall is simpler and more uniform than arbitrary sedimentation rate in mm for columns of varying heights which will yield strikingly different values for the same blood sample depending upon the style of tube employed. None of the ESR values reported here are corrected for differences in packed red cell volume although this correction does not significantly alter the relationships illustrated. The standard Wintrobe chart can be used for this purpose with this method in the same way as employed for the Wintrobe values.

Red cell packed volume. A comparison of this procedure and the Wintrobe in hematocrit determinations on 68 samples of blood covering a wide range of values is illustrated in Fig. 7. It is seen that the 2 methods agree very closely and that 95% of the points lie within the plus or minus 2% boundaries. The difference in required centrifugation time between the 2 methods is shown in Fig. 8. It may be noted that the stationary packing point for a given relative centrifugal force is reached much more rapidly in the larger diameter collection tube and that 5 minutes of centrifugation at 2000 rpm (rcf = 700) is equivalent to the packing obtained in about 30 minutes with the narrow bore hematocrit tube. After 10 minutes of centrifugation the packing value is about 1 or 2% greater than the Wintrobe tube after 45 minutes of centrifugation.

Discussion. The advantages of this method are principally convenience, speed, and economy. Relative accuracy is indicated by Fig. 3, 5, 6, and 7 which illustrate that the obtained values are comparable with a standard current method and distinguish the normal and abnormal categories in the same quantitative manner.

A beneficial result derived from the use of relatively large diameter collection tubes of 10 to 15 mm as compared with 3 mm bore Wintrobe tubes is the substantial increase in speed of the red cell sedimentation. The erythrocyte sedimentation rate is approximately doubled, making it possible to reduce the sedimentation period by one-half, *i.e.*, from 60 to 30 minutes (Fig. 3, 4). The same advantage may be exploited in the packed red cell determination (Fig. 8) where the hematocrit value in the collection tube after 5 minutes of centrifugation is approximately equivalent to the standard Wintrobe procedure at 30 minutes. As a consequence of the various economies, a single technician can run sedimentation rate or hematocrit determinations on several hundred patient blood samples per day. In addition to modification of technical procedure, this method introduces and utilizes the concept of presenting sedimentation rates, as well as hematocrits, as percentages rather than absolute millimeters of fall as commonly expressed (Fig. 6). In the case of the 100 mm Wintrobe hematocrit tube, both mm and percentage criteria are the same, of course.

Since there is no necessity for the transfer of an accurate representative sample, as there is in the usual determination, the danger of sampling error due to improper mixing or measuring is avoided. The additional safety factor of handling a sealed container when testing bloods which may contain infectious agents is a distinct advantage and protection for laboratory personnel. With the use of disposable test tubes there is, of course, no glass washing or hematocrit tube cleaning problem.

Finally, since the blood sample is not used up or contaminated it is available for all other routine determinations which can utilize plasma or whole blood. Bacteriological studies, cellular counts, and many of the blood chemistry determinations may be made from the one blood sample tube(1,6).

Under some circumstances a rapid screening of blood samples for abnormalities is useful. A high degree of accuracy in spotting abnormal specimens may be obtained by taking a 15-minute reading. Values exceeding 10 should be considered abnormal. Final readings should, of course, be made at 30 minutes for confirmation and to obtain standard comparative values.

The proportional volume chart and procedure may also be used for other purposes such as measuring the relative percent of cells in ascitic fluid, the various percentile components of a centrifuged homogenate, sedimented bacteria, chlorella, etc., with no concern for a precise sample volume or calibrated tubes.

Summary. The conventional erythrocyte sedimentation rate and the packed red cell volume procedures have been modified to effect more than a 50% reduction in the operational time required. The essence of the modification consists in determining the sedimentation rates and hematocrit values directly in the original blood collection tube without the necessity of the usual transfer to a secondary, calibrated hematocrit tube. This is accomplished by employing a proportional volume chart which gives the sedimentation rate or hematocrit value for the red cells irrespective of the sample volume fluctuations. All values are expressed as percentages rather than the absolute measurements commonly employed. Erythrocyte sedimentation time is reduced from the standard of 60 to 30 minutes, and hematocrit centrifugation time is re-

duced from 30 to 10 minutes to yield approximately equivalent values. The method has been successfully tested with over 10,000 patient blood samples. The procedure may also be used for similar proportional measurements with ascitic fluid, tissue homogenates, bacterial suspensions, etc., with no need for exact sample volume or calibrated tubes.

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Autoradiography of Carbon-14 Labeled Isoniazid in Brain.* (22258)

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(Introduced by E. M. K. Geiling.)
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The widespread interest in drugs affecting the central nervous system, together with the increasing numbers of isotopically labeled drugs available in research, has stimulated the search for methods of producing autoradiograms of nervous tissue containing compounds labeled with such soft beta emitters as C-14. To date no suitable method has been developed for preparing brain autoradiograms for C-14 containing specimens. Previous methods have been described showing the localization of certain other isotopes not only in individual cells but even in inclusion bodies(1,2). These methods have not, however, been applied to such soft tissues as brain.

The autoradiograms described in this paper, together with supporting differential counting data, show the localization of C-14 labeled isonicotinic acid hydrazide (Isoniazid) and/or its metabolites in gross anatomical structures of brains. C-14 labeled Isoniazid was used as a prototype for these studies because of its known central nervous system effects and because it has previously been shown to localize in the nervous system of mice, rats, and guinea pigs(3). These observations on the localization of C-14 labeled Isoniazid have been confirmed by us in rats and cats, which were used in the autoradiograph studies.

Materials and methods. The Isoniazid used in these studies was synthesized by Murray and Langham of the Health Division, Los Alamos Scientific Laboratories(4). The compound was labeled in the carboxyl position and had a specific activity of 0.031 mc/mg. The labeled drug was diluted with normal Isoniazid in saline to a concentration of 2.5 mg/ml with a resultant activity of 34 x 10^6 dpm/ml. Adult cats were given an intraperitoneal dose of 10 mg/kg of Isoniazid dissolved in normal saline one hour before being anesthetized with pentobarbital sodium. During anesthesia the chest was opened and perfusion with normal saline begun in order to remove the vascular contents from the capillary bed of the brain. The left ventricle of the heart was cannulated and the descending

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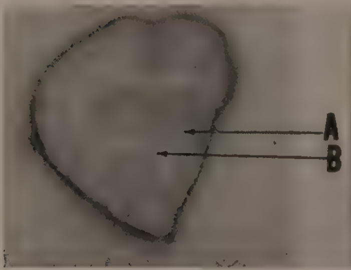

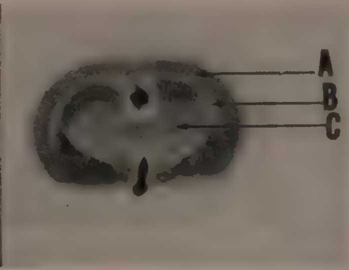

FIG. 1 (left). Autoradiogram of cat brain hemisphere showing density difference between cortex (A) and underlying white matter (B). Peripheral rim of darkest density could represent the very active spinal fluid in subarachnoid space or activity emanating from perpendicular edge of tissue block, or both. Development time, 30 days. Mylar membrane interposed between brain slice and photographic emulsion.

FIG. 2 (right). Autoradiogram of coronal section of rat brain illustrating density difference between cortex (A) and subcortical white matter (B), and showing intermediate density of thalamus (C). Development time, 30 days. No Mylar membrane used. Centrally placed, irregular dark areas represent artifact which is eliminated by the use of Mylar.

aorta was clamped. The right heart was opened to allow return of the perfusate. Two hundred ml of saline was permitted to flow through the system during a period of about 5 minutes. The perfused brains were then carefully removed and placed in a deep freeze to facilitate slicing. Serial coronal sections of approximately 3 mm thickness were made freehand with a 10-inch Valet razor blade, and one hemisphere selected for autoradiography. For C-14 assay purposes, grossly visible anatomical structures were usually selected from the opposite hemisphere, but in some cases it was necessary to obtain additional tissue from the adjacent slice in the same hemisphere. It was found impractical to separate some of the smaller structures (globus pallidus-putamen), and these are represented as single tissues in the accompanying table. Prior to assembly, the tissue slices to be used for autoradiography were placed on a glass slide and chilled to –26°C in a deep freeze, together with black plastic slide boxes (Clay Adams Co., Inc., Cat. No. A1604 B) and foam rubber inserts. The unit was assembled in the dark room by placing Kodak No Screen X-ray film on the floor of the box, covering the free surface of the tissue on the slide with a thin Mylar (du Pont) membrane, and then quickly apposing the tissue and film with the interposed membrane. In our experience the most suitable protective membrane was one quarter mil type A Mylar, which screened out 26% of the beta particles as measured in an open window gas flow Geiger counter. Firm apposition of the tissue slice to the plastic protected photographic emulsion was maintained by placing the foam rubber insert over the inverted glass slide and adherent tissue before closing the slide box. The assembled units thus prepared were sealed with black electrical tape (Minnesota Mining and Mfg. Co. No. 33), and stored at –26°C for 30 days to allow development of the autoradiogram. It is imperative that the dark room operation be carried out rapidly in order to prevent warming of the tissue with resultant softening and distortion due to the slight pressure of the foam rubber. If several autoradiograms are to be prepared at the same time, a pan of dry ice may be used in the dark room to keep the boxes chilled and the tissue slices frozen during the required manipulations. After the 30-day exposure, the film was allowed to come to room temperature before processing. The tissue slices may be fixed in neutral formalin for comparative study.

Results. It may be seen from the illustrations (Fig. 1 and 2) that there is a well defined differential density between various anatomical structures of the brain. It is significant that the relative density of the labeled Isoniazid autoradiograms in the various brain areas is similar to the differential radioactivity of the equivalent anatomical areas as

TABLE I. Activity of C-14 Labeled Isoniazid in Brain Tissues of the Cat. See also Fig. 1. Data by direct plating(6).

Tissue	dpm/mg dry wt
Cerebellar cortex	466
Cerebral "	451
Hippocampus	430
Caudate nucleus	373
Thalamus	380
Cerebral white matter	220
Globus pallidus-putamen	233
Pyramids	175
Brain stem	216
Centrum of brain stem	179
Cerebellar white matter	197
Cerebrospinal fluid	3485*

* dpm/ml.

demonstrated by gross dissection and radioassay (Table I). For example, the cerebral cortex shows an activity of 451 dpm as compared with 220 dpm in the cerebral white matter when calculated on a dpm/mg of dry weight basis. No comparable densitometer measurements of the films were made.

It is to be noted that those areas of localization of C-14 Isoniazid or its metabolites as indicated by the denser areas of the autoradiogram (Fig. 1) correspond to the density of the capillary bed in cortex and white matter of the cat brain, as measured by Hough and Wolff(5). To determine what relationship might exist between drug concentration and vascularity in the central nervous system, time studies with C-14 labeled Isoniazid and other radioactive compounds are in progress. I-131 labeled serum albumin, which is retained within the vascular system, will be used to determine the relative vascularity of various brain areas and the adequacy of the saline perfusion.

Summary. Gross autoradiograms of brain slices containing C-14 labeled Isoniazid, together with supportive data from radioactivity assay, are presented to show the differential localization of Isoniazid in brain tissue, and the technic is described. Future studies to determine the relationship between brain vascularity and brain drug content are indicated.

ADDENDUM: The authors have found that aluminized Mylar gives autograms that are superior to those prepared by using nonmetalized Mylar if more than 30 days exposure time is required.

Aluminized ¼ mil Mylar may be obtained from the Metalized Products Co., Inc., 29 Knight St., Norwalk, Conn.

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Continuous Feed Ligature Carrier. (22259)

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In experiments in our laboratory, where perfusions were made on small animals, ligatures were made around the aorta in 4 places. Since a rather large number of operations were to be performed, a faster method was sought to make these ties. The instrument shown in Fig. 1 was made. In use the needle acts as a probe which once pushed completely under the vessel to be tied supplies the tying thread. The needle is withdrawn and the thread cut with the cutting blade attached to attaching the blade, the needle hub is wrapped with thread, then the blade set in place and wrapped with 21 gauge copper wire. The thread supplies enough springiness so that when the blade becomes dull it can be pushed out and another slid into place on the needle.

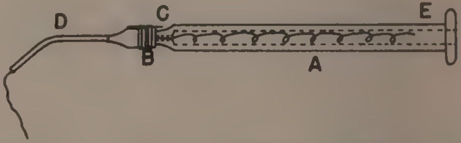

FIG. 1. A, 6 yard coil of 000 surgical silk; B, 21 gauge copper wire; C, steel cutting blade; D, 18 gauge hypodermic needle; E, 1 ml tuberculin syringe.

The thread is wound into a coil as shown in Fig. 2. A motor drives the tapered glass rod for coiling the thread which is held lightly at the small end with the fingers. Six yards of triple zero surgical silk is wound in a coil 10 cm long while moving the string back and forth. Separate turns of the thread should be about ⅛ inch apart. When the coil is fin- the needle. The cutting blade is made from a 0.3 x 1 cm piece of razor blade. Before ished, the side thread B is passed under a previous turn and pulled tight. A glass rod about 1 to 1.5 mm diameter is placed against the end of the thread coiler and the coil pushed onto the rod for storage. When the ligature carrier is to be threaded, the free inside end A is pushed through the syringe and used to pull the coil and rod into the syringe. When all is in the syringe, the glass rod is withdrawn and the thread unwinds freely from the inside of the coil. The instrument is ready to use when the threaded needle is attached.

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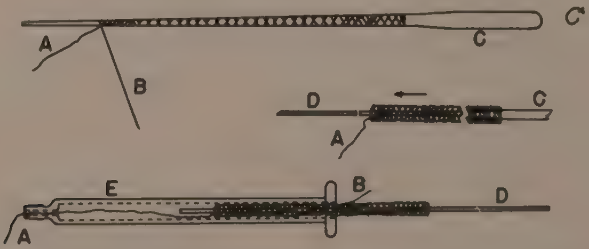

FIG. 2. A, silk thread to inside; B, outside thread; C, thread coiler; D, storage rod; E, 1 ml tuberculin syringe.

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ERRATUM

Article 22147, by Wells *et al.*, Vol. 90, No. 3, page 718, column 2, line 16 should read "glycerol trioleate . . . predominantly unsaturated fat."

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SECTION MEETINGS

CLEVELAND, O.	
Western Reserve University	February 20, 1956
IOWA	
State University of Iowa	January 31, 1956
NEW YORK	
New York Academy of Medicine	February 29, 1956
SOUTHERN	
Tulane University	February 3, 1956

Further Studies of Antigenic Structure of *Pasteurella pestis* in Gels.*

(22260)

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George Williams Hooper Foundation, University of California, San Francisco.

The characterization of the antigens of *Pasteurella pestis* was begun by Schütze(1), who studied them using his serologic absorption and precipitin tests and identified a somatic thermostable and an envelope thermolabile antigen. The double diffusion method of Oudin(2) has provided another means of identifying additional antigens of *P. pestis*—10 by Ranson *et al.*(3), 8 by Burrows(4), 7 by Bhagavan *et al.*(5) and by us. Applying the Ouchterlony method(6) to living culture (7) revealed only 4 precipitation zones, but it was suspected that very small amounts of antigen might have remained undetected due to an excess of antibody in the antiserum used. It was learned that additional antigens do become evident if the culture is first concentrated by fractionation. Using the concentrated extract from *P. pestis* cells or the maximum growth of a shake culture supernatant as antigen in the Oudin test(2) has revealed 3 additional antigens.

Materials and methods. Strains. Those tested were one virulent strain, 195/P, isolated in a case of human plague in India, and 4 avirulent strains—E. V. 76(8,9), TRU(10) from the Java strain Tjiwidej R, TJS(11) and B1456-4(12) isolated by single colony picking from virulent strain B1456. The *P. pseudotuberculosis* strain was the avirulent strain 32, received from Dr. E. Thal, State Veterinary Medical Institute, Stockholm, Sweden. *Antisera.* The highly potent anti-avirulent *P. pestis* gamma globulin was prepared commercially† from serum of rabbits hyperimmunized with living strain A1122. The *P. pseudotuberculosis* antiserum was prepared in this laboratory by hyperimmunizing rabbits with strain 32. *Agar.* A 1.6% Difco bacto-agar was prepared in normal saline and

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† By E. R. Squibb & Sons or Lederle Laboratories.

was sterilized by autoclaving at 15 lb pressure for 15 minutes. *Preparation of antigens.* Each strain was cultured in a casein hydrolysate mineral glucose medium(13) in a 1 liter wide-mouth Erlenmeyer flask containing 250 ml of the medium. The flask was fixed on a shaking machine kept in a 37°C incubation room. The culture was grown for 7 days and was divided according to the demands of the test.

The 5 methods of treating the cultures were (1) adding formalin to make a 1% solution and allowing the mixture to stand at room temperature for 48 hours, (2) adding toluene to make a 1% solution and allowing it to stand at room temperature for 48 hours, (3) placing it in a boiling water bath for 1 hour, (4) treating it with formalin as in (1) and then placing it in a boiling water bath for 1 hour and (5) treating it with toluene as in (2) and then placing it in a boiling water bath for 1 hour. Strains 195/P and B1456-4 were treated by all five methods, and the remaining 3 strains were treated only by the first two methods. The culture was then centrifuged in an angle rotor of a refrigerated centrifuge at 15000 rpm until all of the organisms had been deposited (approx. 30 minutes). The supernatant fluid was carefully removed and was ready for immediate use. *Preparation of tubes* (Oudin method). 1.6% agar in saline with 0.01% merthiolate was melted and maintained at 50°C. 0.7 ml of a 1:2 dilution of anti-A1122 gamma globulin was mixed with 0.3 ml of the melted agar. 0.5 ml of the mixture was delivered into a sterilized tube (8 mm x 80 mm). After the agar hardened, a second layer of 1 ml of 0.8% agar was carefully delivered over the bottom layer with a capillary pipette and allowed to become solid. 0.5 ml of the antigen preparation was delivered onto the top of the second agar layer, and a drop of toluene was added as preservative. The tubes were sealed with rubber stoppers and placed in the refrigerator overnight. The tubes were transferred to a 37°C incubator. Readings were made at the 3rd, 7th and 15th days.

Results. The visible precipitation zones appeared after 3 days of incubation when the antigens and antibodies had diffused into the plain agar layer. The number of zones depended on the antigen preparation used (Table I).

TABLE I. Number of Antigenic Components of *P. pestis* and *P. pseudotuberculosis* Revealed by the Oudin Test.

		No. of precipitation bands	
		Antiserum	
Antigen*	Treatment of antigens	*P. pestis* A1122 gamma globulin	*P. pseudotuberculosis* 32
195/P	Formalin	7	5
B1456-4		7	5
TJS		7	5
E.V. 76		7	5
TRU		7	5
195/P	Toluene	7	5
B1456-4		7	5
TJS		7	5
E.V. 76		7	5
TRU		7	5
195/P	Boiling	3	2
B1456-4		3	2
195/P	Toluene and boiling	3	2
B1456-4		3	2
195/P	Formalin and boiling	1	1
B1456-4		1	1

* 195/P is a virulent strain and the remaining 4 are avirulent.

With *P. pestis* antiserum, the chemically killed virulent and avirulent strains all contained 7 soluble antigens; 4 zones were intense and appeared earlier than the remaining 3. In the Oudin test 3 zones formed in the boiled preparation, 3 in the toluene-killed and boiled preparation and 1 in the formalin-killed and boiled preparation.

When *P. pseudotuberculosis* antiserum was used with the *P. pestis* strains, 5 zones appeared with the formalin or toluene treated, 2 with the boiled and the toluene-treated and boiled, and only 1 with the formalin-treated and boiled.

All of the thermostable antigens of *P. pestis* except the haptenized Fraction I were common to *P. pseudotuberculosis*.

Discussion. When the naturally occurring bacterial cells of *P. pestis* and *P. pseudotuberculosis* strains and their fractions were tested with anti-*P. pestis* gamma globulin by means of a modified Ouchterlony plate technic, 2 antigen-antibody precipitation reactions were common to both organisms, but Fraction I (envelope antigen) and II (toxin) reactions were specific for *P. pestis*(7). The very slight amount of Fraction I in strain 14 and TRU was not revealed, and this was to be expected because the antigens could not reach equivalence concentration.

The Oudin test revealed more antigenic components: All virulent and avirulent strains formed 7 precipitation zones, 5 of which were also common to *P. pseudotuberculosis*. Four of the corresponding antigens in the anti-*P. pestis* serum tube and 2 in the anti-*P. pseudotuberculosis* serum tube were distinctly precipitated. These 4 should therefore be considered major quantitatively. The others more faintly precipitated always appeared slowly.

Of the 7 antigens, 4 were thermolabile and 2, thermostable. The remaining antigen was the haptenized Fraction I. Of the antigens common also to *P. pseudotuberculosis*, 3 were thermolabile and 2, thermostable.

Schütze(1) pointed out that steaming the bacterial suspension in saline for a short time (¼ hour) haptenized the envelope antigen, but that it still precipitated in his precipitin test with its corresponding antibody. Steaming for 1 hour destroys it for *in vitro* reactions. But even after boiling the casein hydrolysate mineral glucose grown culture for 1 hour, the Fraction I (envelope antigen) antigen-antibody reaction zone still was sharply defined in the Oudin test. Boiling for 1 hour haptenizes Fraction I, but it will still form a precipitate with its corresponding antibody. This has been learned by boiling the highly purified Fraction I, in different concentrations, using it as an external reactant.

Also in the complement-fixation test(14) using 4 combining units of the anti-Fraction I serum to react with the supernatant of the boiled and of the toluene-killed and boiled suspension, the reaction has been positive. The complement-fixation test was negative when the formalin-killed and boiled preparation was used.

The fact that 3 zones appeared in the boiled and in the toluene-killed and boiled antigens and that only 1 faint zone appeared in the formalin-killed and boiled antigen indicates that formalin affects the antigens and renders them thermolabile.

The technic of double diffusion in gels is of apparent value for comparative studies of heterologous, but closely related, organisms (7). More than 1 antigen is shared by *P. pestis* and *P. pseudotuberculosis*, and this may account for the cross immunity exhibited between these two species. The Oudin technic confirms that Fraction I is the specific antigen for *P. pestis*. Its toxin has cross-reacted with anti-*P. pseudotuberculosis* serum(15), but in a previous study(7) a slight amount of *P. pseudotuberculosis* toxin was not revealed with the anti-*P. pestis* gamma globulin. The *P. pestis* toxin prepared by the paper electrophoresis method in this laboratory has shown 3 bands with anti-*P. pseudotuberculosis* serum and 3 or 4 bands with anti-*P. pestis* gamma globulin. This might be considered a not highly purified toxin.

Virulent strains were not distinguished from avirulent strains by the test.

Summary. *Pasteurella pestis*, virulent or avirulent, contains 7 antigens demonstrable in the Oudin test. Five are common to *P. pseudotuberculosis*; 3 of these are thermolabile and 2 are thermostable. Of the 7 antigens of *P. pestis* 4 are thermolabile and 2 thermostable. The remaining antigen is haptenized Fraction I. Formalin affects all of the antigens except one, rendering them thermolabile; toluene does not affect them.

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Occurrence of Agglutinogens in Normoblasts.* (22261)

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It is well known that uptake of iron and hemoglobin synthesis occur in the normoblast. There is little information, however, as to the stage of development of the erythrocyte at which agglutinogens first appear. Bjorkman's(1) demonstration that normoblasts may be agglutinated by influenzal virus and a serum containing cold agglutinin suggests that receptors for agglutination make their appearance during this developmental period of the erythrocyte. In his experiments, the normoblasts were isolated from the peripheral blood of a patient with Di Guglielmo's erythro-leukemia; however, the cells were morphologically indistinguishable from myeloblasts. Inasmuch as neoplastic cells could have atypical immunologic characteristics, it seemed desirable to confirm these studies using normoblasts from a patient in which the cells were not of malignant origin.

An opportunity to study agglutinability of normoblasts arose in a patient with autoimmune hemolytic disease secondary to warm agglutinins. About a week following splenectomy, this patient had a hemolytic crisis at which time he released large numbers of nucleated red cells into the peripheral blood. During the period when the normoblast count exceeded 600 per 100 leukocytes, it was a simple matter to isolate them from the peripheral blood for agglutination experiments. A more detailed description of this case will be published elsewhere.

Methods. To isolate the nucleated red cells, 1.0 ml of a 6% solution of dextran in 0.85% saline was added to 20 cc of the patient's freshly drawn blood in a large siliconized test tube. A 10% solution of sequestrene (disodium ethylenediamine tetracetate) was used as anticoagulant. The mixture was incubated at body temperature for 30 minutes. The supernatant plasma, rich in formed elements, was centrifuged at varying speeds, beginning at 600 rpm for 2 minutes. The residue was resuspended in saline and the supernatant plasma recentrifuged at progressively faster speeds. The resulting cell suspensions were checked for cellular content by means of the phase microscope. It was possible in this way to select test tubes which contained a reasonably pure suspension of normoblasts which were then washed 3 times in saline and prepared as a 4% suspension in 0.85% saline. Suspensions prepared in this way contained a minimal number of contaminating erythrocytes and lymphocytes, but no granulocytes or platelets were present. The normoblast suspension was brought in contact with equal portions of serial saline dilutions of serum from the same patient which contained a potent panhemagglutinin, active at 3°C and 37°C. The normoblast suspension was similarly added to serially diluted serum obtained from another patient with a high titer of cold hemagglutinins. The mixtures were incubated for 12 hours at 3°C and read macroscopically and under the phase microscope. The agglutinating effect of nor-

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TABLE I. Agglutination of Normoblasts by Various Sera Obtained from Patient I.B. (Auto-Immune Hemolytic Anemia).

Anti-A serum	P*
Anti-B "	N
Anti-M "	P
Anti-N "	N
Antiglobulin serum	P
I.B. serum (patient)	P
E.S. " (cold agglutinin)	P
E.B. " (" ")	P
Normal A serum	N
B "	P
O "	P

* P = Positive; N = Negative.

mal human sera of various blood groups, in addition to typing sera of the AB and MN and Coombs' antiglobulin serum, were determined following incubation at 37°C for one hour. Siliconized glassware was used in all these experiments.

Results. The reaction of the normoblasts when brought into contact with various sera are listed in Tables I and II. It will be seen that the normoblasts in this patient were agglutinated by anti-A and anti-M serum, which corresponded to his blood type. The normoblasts were strongly agglutinated by antiglobulin serum as were his erythrocytes. Table II lists the titer of serum which resulted in agglutination of the normoblasts. The patient's serum agglutinated his normoblasts in a dilution of 1 to 16 and group O erythrocytes in a dilution of 1 to 8. The serum containing cold agglutinins agglutinated the normoblasts in a dilution of 1 to 256 and normal erythrocytes in a dilution of 1 to 1024.

TABLE II. Titration of Normoblast Agglutinins in Serum of I.B. (Patient) and E.S. (Cold Agglutinin).

	I.B.		E.S.	
Serum dilution	Normoblasts, 3°C	"O" RBC, 3°C	Normoblasts, 3°C	"O" RBC, 3°C
1:1	3+	2+	4+	3+
2	3+	2+	3+	2+
4	3+	1+	3+	2+
8	2+	1+	2+	2+
16	1+	0	2+	2+
32	±	0	1+	2+
64	0	0	1+	2+
128	0	0	1+	2+
256	0	0	1+	2+
512	0	0	±	1+
1024	0	0	0	1+
C	0	0	0	0

Comment. It has been demonstrated in this case that normoblasts derived from a patient with auto-immune hemolytic anemia have antibody receptors which are similar to those of mature erythrocytes. Apparently these receptors make their appearance relatively early in the development of the red cell.

Agglutinability of normoblasts by auto-agglutinins and by antiglobulin serum constitutes direct evidence of the vulnerability of the bone marrow in immune varieties of acquired hemolytic anemia. The severity of anemia in auto-immune hemolytic disease may thus be attributed not only to random destruction of circulating erythrocytes but also may follow a direct attack on proliferating normoblasts. This would limit the ability of the bone marrow to compensate for hemolysis. That impaired erythropoiesis was a factor in hemolytic disease of the new born associated with anti-D antibodies was indicated by the studies of Giblett *et al.*(2). In the case she described, the presence of circulating antibody was correlated with limited erythrocyte production which was resumed following disappearance of anti-D antibodies. In their studies of auto-immune thrombocytopenia, Pisciotta, Stefanini and Dameshek(3) presented morphologic evidence to show that megakaryocytes in a normal recipient could be damaged by transfusion of plasma which contained a strong anti-platelet agglutinin. The damaging effect of the plasma on the megakaryocytes was only temporary and was correlated with a temporarily induced thrombocytopenia.

Summary and conclusions. 1. Normoblasts from a patient with auto-immune hemolytic anemia are agglutinable by type-specific sera, antiglobulin serum and sera containing cold and warm auto-hemagglutinins. 2. Agglutinogens make their appearance at the normoblast stage of development. 3. It is suggested that in acquired immune forms of hemolytic anemia, the bone marrow is also under direct attack by antibodies which may aggravate the severity of anemia.

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Relative Growth-Promoting Activity in Tissue Culture of Co-Factors and the Parent Vitamins. (22262)

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It has been shown(1) that 2 mammalian cells in tissue culture, a human carcinoma cell (strain HeLa) and a mouse fibroblast (strain L), require nicotinamide, pyridoxal, thiamine, riboflavin, pantothenic acid, choline, and folic acid for survival and growth. The degree to which various precursors and conjugates could substitute for the corresponding vitamin in permitting the growth of the mouse fibroblast is here described.

Methods. The technics used in maintaining stock cultures, preparing replicate flasks for feeding with the experimental media, and evaluating the growth response in terms of the number of cells have been described(1-4). As in the previous vitamin experiments(1), specific deficiencies were produced by allowing the cells to grow for varying periods of time (4-14 days) in 1 liter Blake bottles, in the appropriate vitamin-free medium. During this preliminary period of vitamin depletion, the number of cells usually increased 2- to 6-fold in individual experiments; but in most of the experiments, multiplication had then stopped, and there were evidences of cell damage resulting from the vitamin deficiency (cf.(1)). The vitamin-starved cells were resuspended in the appropriate vitamin-free medium, counted, and aliquots of the suspension inoculated into a number of small replicate flasks. Twenty-four hours later, after the cells had adhered to the glass, they were fed with the experimental mixtures containing graded amounts of the vitamin or vitamin substitutes. The flasks were re-fed at 2-day intervals, and the cell count determined after 6-15 days' incubation. Since the cells at the time of the first feeding usually showed cytopathogenic evidence of vitamin depletion, it is apparent that at least 2 factors entered into the results obtained in the present experiments: the capacity of the individual compounds to revive the vitamin-depleted cells, as well as their capacity to support subsequent growth and multiplication. Accordingly, in a second type of experiment, described in the text in relation to folic acid, instead of using vitamin-depleted cells, normal cells were serially propagated in varying concentrations of the vitamin congeners. Diphosphopyridine (DPN) and triphosphopyridine (TPN) nucleotide, flavin adenine mononucleotide (FMN) and dinucleotide (FAD), and cocarboxylase were obtained from the Sigma Laboratories. The first 4 compounds were stated to be 95-98, 60-90, 100, and 65% pure, respectively; but in making stock solutions, all were handled as if they were pure material. Coenzyme A of 75% purity was obtained from the Pabst Laboratory. "Thiamin phosphate" was obtained by autoclaving a solution of cocarboxylase in 0.1 N HCl for 10 min. in a sealed tube. Crystalline pyridoxal phosphate (Palpo) was generously supplied by Dr. E. A. Peterson of the National Cancer Institute, National Institutes of Health.

Results. The results in a number of ex-

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† Public Health Service, U. S. Department of Health Education, and Welfare.

TABLE I. Growth Response of a Mouse Fibroblast (Strain L) to Vitamin Precursors and Conjugates.

	Period of initial starvation in appropriately vit.-deficient medium, days	Degree of cellular multiplication during period of depletion*	Inoculum of vit.-depleted cells, × 10⁴	Period of secondary incubation, days	Degree of multiplication* in media containing indicated conc. (M) of specific vitamin, precursor or conjugate								Approximate max effective conc., M
					10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	0	
Nicotinic acid	10	2.7	25	6	5.2	5.6	3.0	1.0				.7	10^{-6}
Nicotinamide	10	2.7	25	6	2.6	6.2	4.2	.8				.7	"
DPN	10	2.7	25	6	6.0	6.0	2.2	1.1				.7	"
TPN	10	2.7	25	6	3.1	4.3	2.0	.9				.7	"
Pyridoxine†	8	3.1	12	9		8.8	10	10.8	5.7	.3		.97	10^{-8}
Pyridoxal†	8	3.1	12	9		12	10.4	10.2	8.25			.97	"
Pyridoxamine†	8	3.1	12	9		6.7	10.6	5.0	1.0			.97	10^{-7}
Pyridoxal phosphate†	8	3.1	12	9		9.1	6.4	2.4	1.3			.97	10^{-6}
Thiamin	12	4.1	18	8	7.2	9.3	8.6	8.5	5.8	2.0	.6	.55	10^{-8}
Thiamin phosphate	12	4.1	18	8		2.9	3.7	3.7	3.0	1.7	.2		"
Cocarboxylase	12	4.1	18	8	6.0	7.6	7.5	8.6	3.4	1.2	.6		"
Riboflavin	7	.8	14	7				7.6	9.0	2.8		1.1	10^{-9}
FMN	7	.8	14	7		8.0	13.4	7.6	7.9	3.6	1.3	1.1	"
FAD	7	.8	14	7			15.7	9.5	4.0	2.2	1.5	1.1	10^{-8}
Pantothenic acid	7	1.4	18	15	8.3	9.5	10.9	11	.33			0	"
CoA	7	1.4	18	15	7.4	7.9	7.1	.14				0	10^{-7}
Folic acid	14	3.9	16	6		10.6	9.6	4.6	2.0	2.1		.6	10^{-7} to 10^{-8}
Citrovorum factor (natural)†	14	3.9	16	6		11	10.8	10.8	9.2	2.2		.6	10^{-9}†
PABA	14	3.9	16	6		.9		.8		.8		.6	

* Referred to inoculum as 1.

† Gravimetric concentration (g/ml) rather than molar.

periments with the mouse fibroblast are given in Table I. Each experiment there shown is illustrative of several others, with qualitatively similar results.

1. *Nicotinamide,* nicotinic acid, DPN and TPN all permitted the growth of mouse fibroblasts previously depleted of the vitamin by 10 days' starvation. Although the 4 compounds were equally active in terms of their effective concentrations, more growth was usually obtained with nicotinamide and DPN than with nicotinic acid or TPN.

2. *Pyridoxal* and pyridoxine were essentially equivalent in their growth-promoting activity. Although pyridoxamine and pyridoxal phosphate could substitute for pyridoxal, with the preparations here used somewhat higher concentrations were necessary.

3. *Thiamine* and cocarboxylase proved equivalent, both with respect to the concentrations required for maximal growth, and the amount of growth obtained at that optimal concentration. Smaller amounts of growth were, however, observed with "thiamine phosphate."

4. *Riboflavin* and FMN proved equivalent in growth-promoting activity. With FAD, however, significantly higher concentrations were required for a comparable effect.

5. *Pantothenic acid* proved approximately 10 times more active than the 2 samples of coenzyme A used in these experiments (Fig. 1). It is of interest that a similar quantitative difference in the activity of pantothenic acid and coenzyme A was noted by Dewey and Kidder(5) for *Tetrahymena pyriformis* and *Colpidium campylum.*

6. *Folic acid* was regularly less active than natural citrovorum factor in terms of the amounts required for the revival and growth of the vitamin-depleted cells. p-Aminobenzoic acid (PABA) proved wholly inactive (Fig. 1).

To determine whether PABA would permit the survival and growth of normal cells, not previously depleted of folic acid or its derivatives, normal cells, not previously damaged by folic acid depletion, were serially propagated in varying concentrations of folic acid and of PABA. Even under these circumstances, however, PABA failed to permit the continuing growth of the cells in the absence of folic acid.

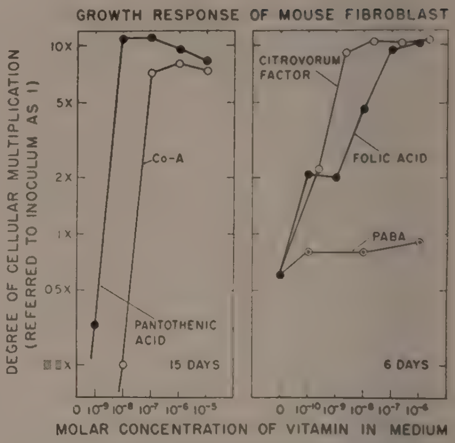

FIG. 1. Varying growth response of mouse fibroblast. (a) Pantothenic acid and coenzyme A; (b) Citrovorum factor, folic acid and PABA.

Summary. A number of the co-factors here tested (FMN, DPN, cocarboxylase) had essentially the same activity as the corresponding vitamin (riboflavin, nicotinamide and thiamine, respectively) in promoting the growth of the mouse fibroblast, in terms of either the effective concentration or the amount of growth obtained. Several vitamin conjugates (FAD, TPN, coenzyme A and pyridoxal phosphate) proved significantly less active than the parent vitamin (riboflavin, nicotinamide, pantothenate, and pyridoxal, respectively). The present experiments provide no information as to the degree to which these differences may reflect only variations in cell permeability, rather than partial blocks in the utilization of these specific co-factors, or in their conversion to a more active form. Natural citrovorum factor, however, was somewhat more active than folic acid. Of the vitamin congeners or precursors here tested, pyridoxine and pyridoxal were essentially equivalent in activity; nicotinic acid was significantly less active than either nicotinamide or DPN in terms of the amount of growth obtained, although the effective concentrations were of the same order of magnitude; while PABA was wholly inactive as a substitute for folic acid. As with the co-factors, the present experiments do not exclude differential cell permeability as the basis of these differences.

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Viral Susceptibility of a Human Carcinoma Cell (Strain KB). (22263)

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The cultivation of a human epidermoid carcinoma of the floor of the mouth (strain KB) by direct implantation in a fluid medium has been described(1). As will be here shown, this cell line has proved susceptible to a number of human and animal viruses.

Methods. The basal medium used in these experiments was the same as that used in the original isolation, and consisted of the amino acids, vitamins and salts essential for the growth of the HeLa cell, at the concentrations optimal for that cell(2-5). In the initial cultivation this was supplemented with 10% whole human serum. In the present experiments, the same basal medium was supplemented with 4% whole horse serum, in order to avoid the complication introduced by the presence in pooled human serum of antibodies to some of the viruses. Four-day cultures of the cell line in T-15 flasks(2), then containing approximately 4.5 x 10^6 cells, were washed twice with the horse serum medium, and refed with 2.5 ml of fresh medium. One-tenth ml of the viral suspensions of Table I, diluted as there indicated, were then added. The medium was then replaced at 2-day intervals. This first passage test was terminated 7 or 8 days after the original inoculation. The times required for the earliest cytopathogenic effect, and for the destruction of more than 75% of the cells, are indicated in Table I. Fluids harvested at the time of maximal cytopathogenic effect (or after 7 to 8 days in the case of those viruses with no apparent effect) were then similarly inoculated into somewhat larger (T-30) culture flasks containing approximately 4.2 x 10^6 cells at time of inoculation. The virus inoculum in this second passage was 0.5 ml in a total of 5 ml. As in the first passage, the fluids were changed every other day, beginning the day after inoculation. The time required for the earliest visible cytopathogenic effect, and for the almost total destruction of the cells is shown for each virus in the last 2 columns of Table I. Material harvested from this second passage at the time of maximal cytopathogenic effect (or after 6 days in the case of those viruses with no apparent effect) was assayed for viral content by either complement-fixation, pathogenicity in tissue culture, or animal inoculation. The results of those tests are summarized in Table II.

Results. Cytopathogenic effects were produced in the KB cell in both first and second passages by type 1 poliomyelitis (Mahoney), herpes simplex, vaccinia, types 1, 3, and 4 adenoidal-pharyngeal-conjunctival (APC) viruses, lymphocytic choriomeningitis (LCM), and encephalomyocarditis (EMC) viruses (*cf.* Tables I and II). In all these, the titers of the second passage fluids indicated that the viruses had multiplied. The titer of complement-fixing antigen produced by the type 1, 3 and 4 APC viruses was significantly higher than had previously been obtained in

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* Public Health Service, U. S. Department of Health, Education and Welfare.

TABLE I. The Susceptibility of the KB Cell to a Number of Human and Animal Viruses.

		Primary inoculation[*]			Second passage[†]		
Virus	Source of virus	Final dilution of viral suspension in culture medium	Days for beginning of cytopathogenic effect	Days for more than 75% of cells to degenerate	Harvested from first passage on day No.	Days for beginning of cytopathogenic effect	Days for more than 75% of cells to degenerate
Poliomyelitis (Mahoney)	Monkey kidney culture	1: 100	1	2	2	1	1
Herpes simplex	Adult mouse brain	"	2	4	4	1	2
Vaccinia	HeLa culture	1: 250	1	2, 3	2, 4, 5 (pool)	1	2
APC, type 1	*Idem*	"	4	6	6	1	2
3	"	"	4–6	>8[‡]	8	1	1
4	"	"	3–4	5–6	6	1	1
Mumps	Suckling mouse brain	1:1000	4	7	7	2	3
"	Allantoic fluid	1: 100	2	3	7	2	3
LCM	Adult mouse brain	1:1000	3	7	7	3	5
EMC	*Idem*	"	1	3	3	6	6
Rabies	Adult mouse brain	1:1000	4	5	7	>6[‡]	—
GD VII	*Idem*	"	6	>7[‡]	7	>6	—
Yellow fever	"	"	5±	>7	7	>6	—
APC (SV_1) (6)	Monkey kidney culture	1: 25	1	1–2	4	>6	—
(DC) (7)	HeLa culture	1: 10	6(?)	>8	8	>6	—
(Type 8) (8)	*Idem*	"	6(?)	>8	8	>6	—
Coxsackie A9	Monkey kidney culture	1: 250	>8[‡]	>8	8	>6	—[‡]
Influenza (type B)	Allantoic fluid	1: 100	>8	>8	7	>6	—
Idem	Monkey kidney culture	"	6(?)	>7	7	>6	—

[*] In T-15 flasks containing 450 × 10^4 cells, and 2.5 ml fluid. Culture fluid changed every 2 days (days #2, 4, 6, 8 in some, and 1, 3, 5, 7 in others) unless cells had degenerated in interim.

[†] In T-30 flasks containing 420 × 10^4 cells, and 5 ml fluid. Culture fluid changed on day #1, 3, 5 (6) unless cells had degenerated in interim.

[‡] No cytopathogenic effect at the time indicated.

HeLa cell cultures. In subsequent experiments, APC viruses of types 1-6 were carried through 5 serial passages in KB cells, and herpes simplex and vaccinia viruses through 3 passages, with rapid production of cytopathogenic effects in all passages. Isolations of all 3 types of poliomyelitis and of several types of APC viruses from field specimens have also been made in KB cells.

Equivocal results were obtained with 4 additional viruses. a) Rabies virus produced cytopathogenic changes in the primary inoculation, but not in the second passage, and no virus could be recovered from the latter. b) The SV_1 strain of APC(6) similarly produced cytopathogenic changes on primary inoculation, and not in second passage. However, the second passage material contained virus, demonstrable by cytopathogenic effect in monkey kidney; and on continued passages through the KB cell, virus was detectable after the fourth passage, indicative of continuing viral multiplication in the absence of significant cytopathogenic effect. c) Both strains of mumps virus produced cytopathogenic effects in both the first and second passage, but did not cause the death of the culture. Cellular degeneration and multiplication proceeded simultaneously throughout the period of test. There is no information as to whether the elaboration of virus in such cultures (*cf.* also SV_1 and yellow fever in this group) reflected the production of virus by only a portion of the cells, which degenerated

TABLE II. Evidence of Viral Multiplication in KB Cell.

Virus	Viral assay of original inoculum*		Viral activity* of 2nd passage harvest				
	Tissue culture	Animal or egg inoc.	Day on which harvested	Estimated dilution of original inoculum in harvest†	Complement fixing titer of antigen	Tissue culture titer	Animal or egg inoculation†
Poliomyelitis (Mahoney)	7.5		1	$1:10^4$		8.5	
Herpes simplex		4.0	2	"			4.8
Vaccinia		‖	3	"		>2.5	
APC, type 1	7.0		3	$1:2 \times 10^5$	1:256	9.5	
" " 3	8.0		1	$1:2 \times 10^6$	1: 32	8.0	
" " 4			2	$1:5 \times 10^5$	1:128	8.0	
Mumps (mouse)		4.5	5	$1:10^8$			>4.0
LCM		5.0	5	$1:10^9$			5.5
EMC		8.6	6	$1:10^6$			>8.0
Yellow fever		6.0	6	$1:10^9$			2.5
APC (SV_1) (6)	6.5		6	$1:2 \times 10^6$		2.5	
Rabies		7.5	6	$1:10^9$			<1.5§
GD VII		7.0	6	"			<1.5§
APC (DC) (7)		‖	6	$1:10^8$	<1:4	<1.0	
" (Type 8) (8)		‖	5	"	"	1.5	
Coxsackie A9	7.0±		5	$1:5 \times 10^8$		ca. 1.5	
Influenza (type B)		7.0	6	$1:10^8$			<1.5§
Idem		3.0	6	"			<1.5§
Mumps (egg)		7.5	5	$1:10^7$			<1.5§

* Logarithm of infectious doses per ml.

† Based on amounts of inoculum introduced into flask, and dilution effected by repeated fluid changes, on the assumption that up to 10% of fluid may have been left each time medium was changed as indicated in footnotes of Table I.

‡ Intracerebrally in adult mice, except for (a) mumps virus, inoculated intracerebrally in suckling mice, and (b) influenza virus, inoculated into allantoic sac of egg embryos.

§ Negative in highest concentration tested.

‖ Original inoculum not titered.

in consequence, or whether even the cells which showed no significant cytopathogenic changes were participating in the elaboration of virus. When the second passage material of the mumps cultures was tested intracerebrally in suckling mice, only the culture of the mouse-derived strain proved infectious; and neither strain was now infectious in eggs. In a following experiment, mumps virus was passaged 5 times. In each passage there was a 5% inoculum, the fluid was changed every 2 days, and the harvest for sub-inoculation was collected on the 6th day. The cytopathogenic effect varied markedly and irregularly for individual passages, from no demonstrable change in the 2nd and 3rd passage, to approximately 50-75% degeneration in the 1st and last passages. The 5th passage harvests were estimated to represent a 10^{-15} dilution of the original inoculum. As in the previous experiment, when the 5th passage material was inoculated intracerebrally in suckling mice, only the culture of the mouse strain was infectious; and neither strain could now produce either infection or demonstrable virus in eggs. There was thus definite multiplication of the mouse-adapted mumps strain; but the only indication that the egg-adapted strain had propagated was the partial and irregular cytopathogenicity. d) Although yellow fever virus produced no significant cytopathogenic effect in either passage, the second passage material proved infectious on intracerebral inoculation in mice. In a second experiment, after 5 passages through the KB cell, estimated to represent a 10^{-15} dilution of the original inoculum, the undiluted culture material produced infection on intracerebral inoculation in mice. As with the mumps strains, the cytopathogenic effects in these serial passages were irregular and incomplete.

The other viruses studied, including mouse encephalomyelitis (Theiler GD VII), 2 other APC strains (type 8 and strain DC), a Coxsackie virus, and influenza type B, produced no cytopathogenic effects in either passage, and except for a small amount of residual virus in the second passage of the APC type 8 and Coxsackie A9, there was no evidence of viral multiplication. In subsequent attempts with higher titer inocula, type 8 APC has been established in serial passage. It should be pointed out that the DC strain grows poorly in all cell types tested in tissue culture, including HeLa cells(7).

Summary. 1) A human epithelioid carcinoma cell in tissue culture (strain KB) has been found susceptible to a number of viruses, including poliomyelitis, herpes simplex, vaccinia, a number of APC strains, LCM and EMC. Marked cytopathogenic effects were produced in 1-7 days, and viral multiplication was shown by complement fixation, tissue culture, or animal titration of the second passage material. 2) Only partial and irregular cytopathogenic effects were obtained with mumps, yellow fever, and the SV_1 strain of APC virus, but with apparently continuing elaboration of virus on repeated passage. Both an egg-adapted and a mouse strain of mumps virus were found to have lost their infectivity for eggs after several culture passages. 3) Coxsackie virus (strain A9), influenza type B, one APC type, and GD VII had no cytopathogenic effect on this cell, and there was no evidence of viral multiplication. Rabies virus was cytopathogenic in the first passage only; and the harvest of the second passage was not infectious on intracerebral inoculation in mice.

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Comparative Absorption of Vitamin B_{12} Analogues by Normal Humans. II. Chloro-, Sulfato-, Nitro- and Thiocyanato- *vs* Cyanocobalamin. (22264)

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The first(1) paper of this series reported results of comparative oral absorption studies of chlorocobalamin and cyanocobalamin employing the urinary excretion method of Schilling(2). These tests were interpreted as indicating the superiority of cyanocobalamin over chlorocobalamin for alimentary absorption by humans. Results of oral tolerance tests with humans, and of fecal excretion measurements with rats, confirming the low chlorocobalamin absorption, are reported herein. In addition, similarly inferior absorptions of sulfato-, nitro- and thiocyanatocobalamins are reported, based upon urinary and fecal excretion studies in humans, and upon fecal excretion by rats.

Methods. Materials. The preparation and properties of the radioactive and normal chlorocobalamins and cyanocobalamins employed in these experiments have been described(1). Sulfatocobalamins were obtained photochemically as for chlorocobalamin except that irradiation was performed in 0.01 M H_2SO_4 solution. Samples of the nitroco-

TABLE I. Urinary Excretion of Radioactivity by Humans.

Cobalamin			Avg % of dose ± stand. dev.
Oral		Inj., 1 mg	
Cyano	2 μg	Cyano; chloro	≃12*
Sulfato	"	Sulfato	3.5 ± 1.5
		Chloro	2.6 ± .8
		Cyano	3.7 ± 1.8
Nitro	"	Nitro	2.0 ± .8
		Chloro	3.1 ± .5
		Cyano	1.9 ± .2
Thiocyanato	"	"	2.0 ± .9
Nitro	.5	"	5.8 ± 3.6
Cyano	"	"	17.6 ± 9.0

* Avg of accumulated standard test data.

balamins and thiocyanatocobalamins were prepared from sulfatocobalamins by treatment with acid nitrite and KSCN respectively. Paper chromatograms,* distribution coefficients and absorption spectra attested to the identity of the radioactive and normal forms. Specific activities of the radioactive cobalamins were: cyano-≃200 μc/mg; sulfato-≃130 μc/mg; nitro-≃130 μc/mg and thiocyanato-≃171 μc/mg. The urinary excretion(2,3) and the Heinle-Welch(4) fecal excretion (5-6 days) methods employed to compare the several labeled cobalamins have been adequately described elsewhere. In all cases the subjects (groups of 4-6) were young, healthy adult males under continual supervision to insure complete collection of excreta. Urine and feces radioactivity was determined by scintillation counting. Two series of oral tolerance tests were performed with chloro- and cyanocobalamin as described by Chow(5). Subjects were fed 3 or 5 mg of test substance in aqueous solution after first removing 15 ml of fasting blood for microbiological assay of serum for vitamin B$_{12}$. Two hours after ingestion, another 15 ml sample of blood (still fasting) was removed for vit. B$_{12}$ assay. Aged subjects, *i.e.* over 65 years, were employed in order to reveal more readily resultant changes in serum vit. B$_{12}$. Only differences in excess of 40 $\mu\mu$g/ml are considered experimentally significant(5).

Normal adult male rats of the Johns Hopkins colony, weighing an average of 300 g per animal, were employed (in groups of 6) in fecal excretion experiments with cyano-, chloro- and nitrocobalamin-Co^{60}. A 50 mμg dose of labeled analogue was fed to each rat by stomach tube and feces collected for 5 days. All collections were measured for cobalt 60 content by scintillation counting of feces homogenates. Actually, excretion of radioactivity was negligible after the first 24-48 hours.

Results. Human urinary excretion results are compiled in Table I. Table II contains the results of 2 fecal excretion studies with 0.5 μg of cyano- and nitrocobalamin-Co^{60} administered to human subjects. Although daily measurements were performed, only cumulative excretions for the entire collection periods are of interest. Group average cumulative excretions measured, and average absorption values computed therefrom by difference, are reported.

Results of the 2 oral tolerance tests are compiled in Table III. Initial concentrations and maximum difference, observed after 2 hours, are reported in terms of $\mu\mu$g/ml of serum.

TABLE II. Fecal Excretion of Radioactivity by Humans.

Study	Cobalamin (.5 μg oral)	Avg % excr. ± stand. dev.	Avg % absorbed
1st* (5 days)	Cyano	18.0 ± 7.1	82.0
	Nitro	41.8 ± 12.8	58.2
2nd (6 days)	Cyano	13.7 ± 4.4	86.3
	Nitro	46.5 ± 6.9	53.5

* 1 mg vit. B$_{12}$ injected 2 hr after oral dose of tracer.

TABLE III. Serum Levels ($\mu\mu$g/ml) in Humans.

	Cyano		Chloro	
Study	Initial	Difference (2 hr)	Initial	Difference (2 hr)
1st	280	682	222	0
3 mg cyano;	146	854	216	0
3 mg chloro	251	70	157	0
	169	286	642	0
	227	94	570	305
2nd	99	204	169	76
3 mg cyano;	122	158	140	41
5 " chloro	157	141	251	46
	227	94	490	0
	198	64	146	0

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* The authors are grateful to R. D. Babson of the Research Laboratories of Merck & Co., for these chromatographic tests.

TABLE IV. Fecal Excretion of Radioactivity by Rats.

Cobalamin (.050 μg oral)	Avg % excr. ± stand. dev.	Avg % absorbed
Cyano	65.4 ± 7.2	34.6
Chloro	90.6 ± 9.5	9.4
Nitro	85.2 ± 15.2	14.8

Table IV is a summary of the findings from the fecal excretion study with cyano-, chloro- and nitro-cobalamin-Co^{60} administered to rats. Here too, group average cumulative excretions (after 5 days) and computed absorptions, are reported.

Discussion. Application of the urinary excretion method at 2 μg to sulfato-, thiocyanato- and nitrocobalamin reveals lower responses than are obtained with cyanocobalamin, as can be seen from the summary Table I. For nitrocobalamin, this is true also at the 0.5 μg oral level. The low sulfato-excretions (≃3% of the 2 μg dose) are practically identical with those reported previously for the chloro-analog, and are about one-fourth the average 12% figure typical of vit. B_{12} itself. Similar behavior characterizes nitrocobalamin and thiocyanato cobalamin *i.e.* low responses of 1.9-3.1% of the 2 μg dose independent of the choice of injected analogue. Even at 0.5 μg oral intake, the urinary nitrocobalamin output of 5.8% is much less than the 17.6% elimination of cyanocobalamin. Furthermore as in the case of labeled chloro-(1), the nature of the injected cobalamin is immaterial. Thus sulfato-, chloro-, nitro- and cyano-cobalamin are indistinguishable in their capacity to induce cobalamin excretion upon injection, which again points to the equivalence, perhaps through interconvertibility of cobalamins or conversion to another common structural analogue, of the several cobalamins once introduced into the blood stream.

The similarity in behavior of the chloro- and sulfato-forms is understandable in terms of their identical electrolyte and hydrophilic properties(6,7) and spectra. Less evident is the reason for the slight responses obtained with nitrocobalamin and thiocyanato-cobalamin. These compounds are characterized as electrically neutral cobalamins(7,8) since the substituent anions are relatively tightly bound(9) and because of their relative organophilic tendencies(9,10). In this respect they resemble cyanocobalamin rather than aquocobalamin salts, and might have been expected to exhibit a pronounced absorption closely approximating that of vit. B_{12} itself. Despite evidence for slight dissociability in certain cases, addition of cyanide ion converts all known cobalamins to the cyano form. The current results then point to the unique importance of the latter group in governing the metabolic activity of the cobalamins, rather than to the dissociability of the substituent anion. It is conceivable that even the low non-cyano responses are themselves due to partial interactions with traces of cyanide ion in gastric juice or upper intestinal contents rather than to an inherent activity of these cobalamins.

That the extent of urinary excretion after injection is an index of absorption has been verified by independent methods of demonstrating absorption.† In the case of chlorocobalamin, oral tolerance tests (Table III; first study) with humans failed to reveal any increase in serum cobalamin concentration in four out of five subjects fed 3 mg doses; and even feeding of 5 mg (Table III; second study) produces a barely significant rise in 3 out of 5 persons. By contrast, ten individuals receiving 3 mg of cyanocobalamin each exhibited serum levels 2 hours after ingestion which were on the average approximately 2-3 times normal. Further evidence for the superior absorption of cyanocobalamin is provided by the fecal excretion measurements with rats. It is obvious from Table IV that the absorption (9.4% of the 50 mμg oral dose) of chlorocobalamin is only about one-fourth the 34.6% absorption typifying cyanocobalamin.

Similar confirmation has been obtained in studies comparing the fecal excretion of labeled nitrocobalamin with that of cyanocobalamin by rats and humans. In the rat studies (Table IV) nitrocobalamin absorption (14.8%) is less than half the 34.6% value obtained with the cyano- form. In the first of the human studies (Table II), fecal excretions of nitro- and cyano- forms were respectively 41.8% and 18.0% of the 0.5 μg oral dose which presumably correspond to absorptions of 58.2% and 82.0%. Respective absorption figures of 53.5% and 86.3% obtained in the second study are in excellent agreement. When one considers that "zero" absorption values, as are obtained with pernicious anemia patients(12), may range from 0 to 24% (average$\simeq$12%), these figures would indicate a 2 to 1 superiority in absorption in favor of cyanocobalamin.

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† G. B. J. Glass, N. Y. Medical College has observed(11) by hepatic uptake method that the cpm of liver projections after oral administration of 0.5 μg of chlorocobalamin-Co^{60} is only one-half to one-third the radioactivity noted after administration of cyanocobalamin-Co^{60}. This is in accord with conclusions presented in our paper.

It is significant that all methods support the observations, based upon the urinary excretion method, that cyanocobalamin excels the other cobalamins as an oral absorption form. One may conclude further that, as regards the cobalamin family, the injection-excretion test can serve as an index by which to judge comparative absorption.

Summary. 1. Urinary excretion measurements indicate considerably lower oral absorption of sulfato-, nitro- and thiocyanatocobalamin than is exhibited by cyanocobalamin. In the blood stream, however, the several cobalamins are equivalent in inducing the excretion of ingested vit. B_{12}. 2. That cobalamin urinary excretion responses truly constitute an absorption index was verified in studies comparing chlorocobalamin with cyanocobalamin, by serum level assays in humans and by fecal excretion experiments with rats. 3. Inferior absorption of orally administered nitrocobalamin was confirmed by fecal excretion observations in humans and rats. 4. The superior absorption of cyanocobalamin (vit. B_{12}) over other cobalamins studied is attributed to either the chemical specificity of the substituent cyano group or the tightness of its binding in the cobalt coordination sphere.

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Decrease of Coenzyme A Content in Fatty Liver.* (22265)

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Some years ago it was found that pantothenic acid deficiency produces fatty liver in dogs(1). This observation appeared of particular interest after the discovery of coenzyme A (CoA)(2) and the identification of pantothenic acid as precursor of CoA(3,4), this coenzyme having a pivotal position in degradation and synthesis of fatty acids and cholesterol(5-7). Since CoA represents the functional form of pantothenic acid in tissues, it appeared reasonable to suppose some relation between CoA-deficiency and formation of fatty liver. However, more recently it has been reported that, not only does fatty liver not occur in pantothenic acid-deficient rats but that these rats are resistant to liver fat deposition ordinarily caused by cholesterol-rich diet(8). The difference of results in the 2 species is probably due to the fact that adrenal changes produced by pantothenic acid deficiency are much more severe in rats than in dogs(*cf.* 9). Adrenal hypofunction has been found to occur in pantothenic acid-deficient rats, which, in some respects, react as though adrenalectomized(10). That adrenal glands play an important role in the formation of fatty liver is shown by the fact that adrenalectomy prevents deposition of fat in the liver due to different steatogenic treatments(11-16). It seemed to us worthwhile to investigate whether CoA is implicated in the pathological accumulation of fat in liver. This paper reports the results of the estimation of CoA in fatty liver produced with carbon tetrachloride.

Materials and methods. Adult female albino rats, weighing between 250-300 g, were used. Animals were kept on a standard diet of the following composition: glucose, 73%; casein, 18%; maize oil, 3%; cod liver oil, 2%; salts, 4%(17). Supplementary crystalline vitamins were added in the following quantities per 100 g of diet: thiamine, 400 μg; riboflavin, 800 μg; pyridoxine, 400 μg; niacin, 2000 μg; calcium pantothenate, 2000 μg and choline chloride, 100 mg. After 3 weeks on this diet, the animals were divided into 2 groups. Rats of the first group were used as normal controls and those of the second group were injected for 8 days with CCl_4 to produce fatty liver (0.2 ml/100 g body wt/day of 20% solution of CCl_4 in olive oil, subcutaneously). During treatment with CCl_4 the animals were fed with the aforementioned standard diet. Both normal and experimental rats were fasted for 12 hr before the experiment. They were killed by decapitation and livers were quickly removed. Portions of liver were taken for histological examination and for estimation of nitrogen and lipid contents. The remainder of hepatic tissue was immediately chilled and homogenized with water. The homogenate was rapidly boiled, centrifuged, filtered(18) and CoA estimated in the boiled extract by a modification(19) of the method of Kaplan and Lipmann(18). The tissue samples for histological study were placed in 10% formalin within a few minutes after death; paraffin sections were stained with hematoxylin-eosin and frozen sections were stained with Sudan III for fat and hematoxylin as a counterstain. Nitrogen was determined with Nessler's reagent after digestion of the samples with sulphuric acid containing copper selenite(20). Total lipids were determined by extraction of dry tissue with ether in Soxhlet apparatus and weighing of fatty residue after removal of solvent. Tissue was dried by heating in oven at 100°C until constant weight. Acetylating apoenzyme was prepared from pigeon livers according to Kaplan and Lipmann(18) but livers were frozen overnight and homogenized directly from the frozen state into cold acetone as suggested by Handschumacher, Mueller and Strong(19). Chromatographically pure 4-aminoazobenzene was used; the commercial product ob-

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TABLE I. Coenzyme A Content in Normal and Fatty Livers.[*]

	Total lipids, mg/mg liver N.	CoA, μg pantothenic acid bound as CoA/mg liver N.
Normal rats	1.32 ± .07	2.2 ± .13
CCl_4-intoxicated rats	2.41 ± .11	1.54 ± .11
Difference of the means	1.09	.66
P[†]	<.01	<.01

[*] Figures are means of 8 experiments ± stand. error.

[†] Probability determined by *t* test.

tained from Eastman Kodak Co. was purified as indicated by Handschumacher *et al.*(19). Adenosine triphosphoric acid (barium salt) and a crude preparation of CoA were obtained from the Sigma Chemical Co. All the other chemicals were reagent grade.

Results. Microscopic examination confirmed that CCl_4-intoxicated rats developed typical fatty livers. Chemical analyses of livers revealed amounts of lipid which corresponded well with the microscopic picture. As to be expected, the fat content was markedly increased in livers of CCl_4-intoxicated animals (Table I). The results of CoA estimations in both normal and pathological livers are summarized in Table I. It is apparent that CoA content was significantly lowered in fatty livers as compared with normal controls (30% decrease).

Discussion. Results presented are sufficient evidence for a marked decrease of CoA content in fatty liver. However, because of the method used in estimation of CoA it is possible that the actual decrease of CoA in fatty liver was greater than we observed. It has been shown that pigeon liver extract of Kaplan and Lipmann(18), in the presence of adenosine triphosphate (ATP) is capable of synthesis of CoA from some fragments containing pantetheine, in particular from dephospho-CoA and from phosphopantetheine (21,22). The CoA-assay system used in our experiments included both ATP and the Kaplan-Lipmann extract of pigeon liver as an acetylating apoenzyme. Thus, values for CoA here reported represent the sum of preexisting complete CoA plus any CoA synthesized from smaller molecules during the assay. The possibility exists that the amount of precursors of CoA was increased in fatty liver because of an impaired formation of complete coenzyme. If this were true, the synthesis of CoA during the assay would erroneously raise the figures for fatty livers more than those for normal livers. The uncoupling of oxidative phosphorylation found in mitochondria from fatty livers(23) could account for the impairment of CoA synthesis and for the accumulation of the precursors. ATP, in fact, is involved in biosynthesis of CoA(24). It is also to be noted that mitochondria swollen *in vitro* release CoA into the suspension medium where the coenzyme is broken down to fragments containing pantetheine(25). This observation may be of interest because mitochondria from fatty liver closely resemble, in their morphology and enzymatic activities, osmotically damaged mitochondria(23). Although an impaired synthesis seems the most likely explanation for the decreased content of CoA in fatty liver, the possibility of an increased breakdown of the coenzyme cannot be ruled out. It also remains obscure whether the CoA-deficiency plays a pathogenic role in the formation of fatty liver or it only represents a consequence of cell damage. As far as concerns the possible participation of CoA-deficiency in the pathogenesis of fatty liver, a hypothesis can be put forward if only provisionally. The low level of CoA and ATP, which are necessary for activation of fatty acids, would lead to impaired utilization of lipids in the liver and, consequently, to pathological accumulation of fat. Of course, this pathological condition is more severe when fat mobilization from depots to the liver is accelerated. According to the results of previous work(*cf.* 26), an increased mobilization of fat from the peripheral tissues to the liver actually occurs during fatty liver formation.

Summary. Coenzyme A has been estimated in both normal and CCl_4-fatty livers. The level of coenzyme A is decreased in fatty livers as compared with normal controls. The possible role of this change in the formation of fatty liver is discussed.

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Determination of L-Glutamic Acid by Use of L-Glutamic Acid Decarboxylase from *Mycobacterium phlei*. (22266)

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Amino-acid decarboxylases of bacterial origin are now widely used for the quantitative estimation of certain amino-acids(1-7,9). Gale(1) suggested the use of *Clostridium welchii* SR-12 for the determination of glutamic acid. This organism releases CO_2 not only from L-glutamic acid, but from L-glutamine(3), and L-aspartic acid(5,6) as well. Additional tests and special conditions are necessary(3,5,6) to obtain separate values for each of these compounds. Glutamic acid decarboxylases obtained from different strains of *E. coli* were used by several investigators(2,7-9) for the assay of L-glutamic acid. In general, the *E. coli* preparations possessed in addition to L-glutamic acid decarboxylase, a glutaminase(7), or a L-lysine and L-arginine decarboxylases(2,8,9). Recently, Najjar and Fisher(9) reported that they were able to destroy the arginine and lysine decarboxylases without seriously affecting the L-glutamic acid decarboxylase.

In the present paper a description of the use of cell-free extracts from *Mycobacterium phlei* for the determination of L-glutamic acid will be given. Extracts from this organism decarboxylate quantitatively L-glutamic acid only and do not react with 18 other amino-acids tested. Furthermore, no glutaminase or glutamo-racemase activity could be detected under conditions optimal for the decarboxylase activity.

Methods. A strain of *Mycobacterium phlei* from the stock of this laboratory is grown in Roux bottles on nutrient agar medium (peptone—1%, meat extract "Bovril" —0.3% • NaCl—0.50%, $Na_2HPO_4 \cdot 12H_2O$ —0.25%), to which 0.1% Tween 80 was added, and incubated for 3-4 days at 35°C. The total crop from 6 bottles is washed 3 times with saline, resuspended in 20 ml distilled water and then disintegrated in a 9 KC Raytheon sonic vibrator for 25 minutes. An alternative method for preparing the extracts by grinding the bacterial paste with alumina A-301, according to McIlwain(10) was also used and proved quite satisfactory. After disruption of the cells the debris are removed by a Servall centrifuge at 10,000 r.p.m. for 20 minutes and the clear and somewhat opalescent supernatant is dialyzed against running tap water in the cold-room for 13-15 hours. The protein content of the dialyzed solution is determined by Mehl's Biuret method(11).

Results. Measurements of glutamic acid decarboxylation. a). The Warburg manometric technic is used and from the amount of CO_2 evolved the quantity of glutamic acid that underwent decarboxylation can be calculated. Suitable controls containing no substrate and other controls containing substrate with KOH in the central well are run simultaneously with the complete system. All measurements are made at 37°C in citrate-phosphate buffer, pH 5.2, final concentration as indicated by McIlvaine(12), with air as the gas phase. The main compartment contains the enzyme and the buffer; the substrate is tipped in from the side-arm after equilibration for 20 minutes. For estimation of the reaction rate measurements are taken every 5-10 minutes. For quantitative estimation of glutamic acid readings are made until no changes in gas pressure are seen. The recovery of glutamic acid is 95-98% and owing to the low pH of the system there is hardly any retention of CO_2 by the liquid phase. b) Paper chromatography was used as an alternative method for the quantitative determination of the other product of decarboxylation of glutamic acid—γ-amino-butyric acid. Circular chromatography after Giri *et al.*(13) was employed; the solvent used was water-saturated phenol. The effect of pH on the activity of the enzyme is presented graphically in Fig. 1. The optimum activity is at pH 5.2-5.4, falling off rapidly at both sides of the pH curve, the slope being even steeper on the alkaline side.

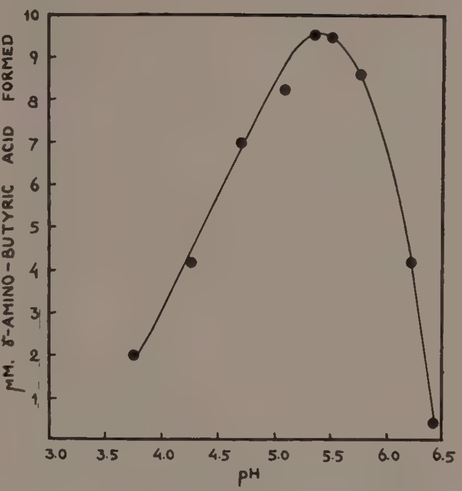

FIG. 1. The system contained: 10 μM L-glutamic acid, cell-free extract equivalent to 6.8 mg protein, McIlvaine's buffer, pH values as indicated, total volume—1 ml; time of incubation—2 hr at 37°C. The γ-amino butyric acid was determined by quantitative paper chromatography(13).

The influence of enzyme concentration on the reaction rate was studied and as is appar-

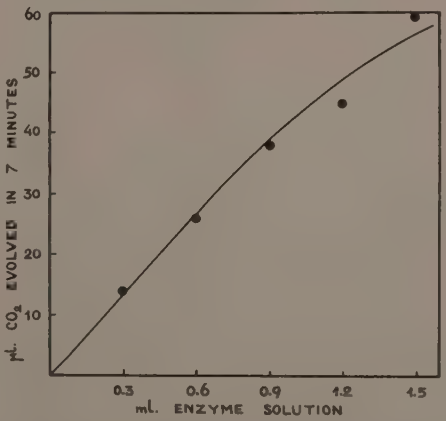

FIG. 2. The system contained: 10 μM L-glutamic acid, varying quantities of a cell-free extract (7.5 mg protein/ml), McIlvaine's buffer—pH 5.2; total volume—2.5 ml. Incubation at 37°C.

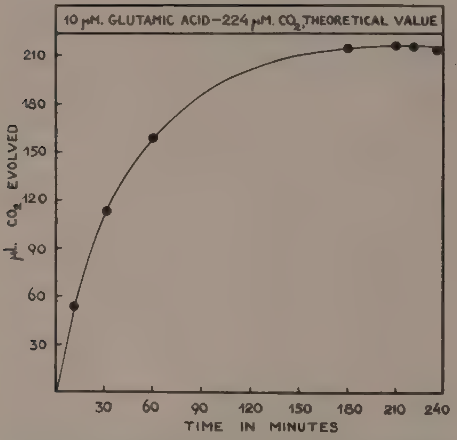

FIG. 3. The system contained: 10 μM L-glutamic acid, cell-free extract equivalent to 15 mg protein, McIlvaine's buffer—pH 5.2; total volume—2.5 ml. Incubated at 37°C.

ent from Fig. 2, activity is proportional to enzyme concentration as long as the enzyme is saturated with substrate. For the assay the enzyme is always taken in excess (15-20 mg protein per vessel). A typical experiment is illustrated in Fig. 3. Dialysis of the enzyme solution is not essential. However, when undialyzed extracts were used, the blanks showed considerably higher values. Only slight differences in activity (about 10%) were noticed when dialyzed and undialyzed extracts were compared. The enzyme preparation is quite stable; about ⅔ of activity were preserved after storage at 0°C for 4 months.

Specificity for L-glutamic acid. In repeated experiments with a series of amino-acids the cell-free extracts from *Mycobacterium phlei* did not decarboxylate any amino-acid other than L-glutamic acid. The amino-acids tested were: L-alanine, L-arginine, L-aspartic acid, D-glutamic acid, L-glutamine, glycine, DL-histidine, DL-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-ornithine, DL-proline, DL-serine, DL-threonine, DL-tyrosine, L-valine. Furthermore, there is no appreciable change in decarboxylation rate of L-glutamic acid when D-glutamate, L-aspartate, L-glutamine, or L-asparagine are present. (Table I).

Partial purification of enzyme. A cell-free extract was prepared as already described. Ammonium sulphate was added gradually to obtain 33% saturation. The precipitate (fraction I) was centrifuged off and to the clear supernatant a further amount of ammonium sulphate was added until 50% saturation was obtained; centrifugation was then repeated and after removing the precipitate (fraction II) more ammonium sulphate was added to the supernatant until 70% saturation was reached. The precipitate (fraction III) was removed by centrifugation. All the manipulations were carried out in the cold-room. Table II shows the activities of the various fractions (after dialysis to remove the $(NH_4)_2SO_4$) as tested for glutamic acid decarboxylase. Fraction II (precipitate from 33-50% saturated $(NH_4)_2SO_4$ solution) contained over 50% of the original activity with a 3-4-fold purification.

Discussion. The enzyme preparation described in this paper has the advantage of being highly specific towards L-glutamic acid; none of the other 18 amino-acids tested is attacked by the enzyme. Closely related amino acids and amides (D-glutamic acid, L-glutamine, L-aspartic acid and L-asparagine) did not interfere with the activity of the glutamic decarboxylase. The lack of any measurable glutaminase activity enables direct estimation of L-glutamic acid in natural

TABLE I. Influence of Related Substances on Decarboxylation of L-Glutamic Acid.

Substrates (μM)	CO_2 evolved* (μM)	γ-amino butyric acid formed* (μM)
L-glutamic acid, 10	.60	.60
D-glutamic acid, 10		.0
L-glutamine, 10	.0	.0
L-aspartic acid, 10	.0	.0
L-glutamic acid, 10 + D-glutamic acid, 10		.58
L-glutamic acid, 10 + L glutamine, 10	.60	.58
L-glutamic acid, 10 + L-aspartic acid, 10		.62
L-glutamic acid, 10 + L-asparagine, 10		.62

* Values calculated/mg enzyme protein/hr.

The reaction mixture was set up in McIlvaine's buffer, pH 5.2, in a total volume of 1 ml, and incubated at 37°C; the γ-amino butyric acid formed was determined by quantitative paper chromatography(13).

TABLE II. Partial Purification of L-Glutamic Acid Decarboxylase with Ammonium Sulfate.

	Total activity (units)*	Total protein (mg)	Specific activity (units per mg protein)
Crude extract	216	1200	.18
F I .33 s†	46	760	.06
F II .50 s	113	180	.63
F III .70 s	5	40	.12
Supernatant at .70 s	0	213	.0

* 1 unit is defined as that amount of enzyme which will decarboxylate 1 μM of glutamic acid in 10 min.

† F = fraction; s = saturation.

The tests were performed with dialyzed enzyme samples; the γ-amino butyric acid formed was determined by quantitative paper chromatography.

products containing considerable amounts of glutamine. The cultivation of the bacteria and the preparation of the extract do not present any difficulties. It is possible to prepare a larger amount of extract and preserve it for a long period by cold-storage.

Summary. 1. A preparation from *Mycobacterium phlei* with decarboxylase activity towards L-glutamic acid only, was described. The preparation has no detectable decarboxylase activity towards other 18 amino-acids tested, and no glutaminase or glutamo-racemase activities. This enables direct estimation of L-glutamic acid in natural products. 2. The optimal pH range is between pH 5.2-5.4, with a sharp drop in activity at pH values of 4 and 6 respectively. 3. Some purification of the enzyme was achieved by fractionation with ammonium sulphate.

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Interrelationships of Glucose and Inorganic Phosphorus in Blood and Urine of Patients with Diabetes Mellitus.* (22267)

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Studies in relationships of inorganic phosphorus and glucose in blood, both in experimental animals and in man, have contributed to the development of present-day concepts concerning the role of phosphorus in metabolic processes(1). It has been established that marked lowering of blood glucose following administration of insulin is associated with simultaneous fall in phosphorus in blood and urine(2,3). A similar decline in inorganic phosphate also occurs following administration of glucose(4-6). The fall in serum phosphate has been shown to be related to active entry of glucose into the glycolytic cycle in muscle, rather than into the glycogeneic cycle in the liver(7). These observations have been extended and related to the action of insulin, epinephrine, and certain hypophyseal hormones(8). On the basis of these findings, the fall of serum inorganic phosphorus following intravenous administration of glucose has become an accepted criterion for peripheral utilization of glucose.

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TABLE I. Summary of Pertinent Data and Correlation Coefficients between Inorganic Phosphorus and Glucose.

Patient	Blood glucose, range, mg %	Plasma inorganic P, range, mg %	Urine glucose, range, g/24 hr	Urine inorganic P, range, g/24 hr	Days observed	Correlation coef. between blood glucose and plasma inorg. phosphorus	Correlation coef. between urine glucose and urine inorganic phosphorus
J.F.	36-468	3.0-5.2	.5- 90.8	.45- .90	33	−.73†	−.20
R.E.	90-211	2.8-3.5	.3- 11.9	.41-1.19	37	−.27	.21
F.H.	96-451	3.0-4.4	.8-140.0	.40-1.22	22	−.20	.58†
E.M., '53	87-368	3.6-4.7	5.1-116.0	.72-1.31	12	−.68*	.51
'52	60-367	1.8-3.7	—	—	32	−.62†	—
M.K.	53-442	3.4-5.0	—	—	36	−.26	—
J.M.	74-325	3.4-5.0	.4- 33.5	.43-1.42	24	−.15	.27
M.W.	50-331	2.4-4.4	.8- 42.7	.29- .79	32	−.69†	.37*
H.S.	46-356	1.9-4.2	—	—	31	−.15	—
M.A.	88-163	3.4-4.9	2.9- 29.3	.83-1.18	6	−.96†	.06
G.C.	145-473	1.8-4.4	—	—	23	−.28	—
G.M.	44-540	2.7-3.9	.2-144.9	.86-1.72	26	−.46*	.33
M.S.	44-369	2.9-5.0	.6- 83.5	.40-1.17	30	−.68†	.30
J.B.	30-244	2.2-5.0	—	—	33	−.43*	—
L.F.	75-353	2.6-5.0	1.0- 85.3	.42-1.26	42	−.44*	.18
S.D.	57-267	3.5-5.6	.7- 9.3	.78-1.20	11	−.72*	−.07

* $P<.05$. † $P<.01$.

Clinically, changes in serum inorganic phosphorus during intravenous glucose tolerance test have been used to differentiate diabetes mellitus from hyperglycemia of liver disease and other syndromes(9-12).

With these concepts in mind we set about to determine the relation of blood phosphorus to spontaneous and induced changes in blood sugar levels in patients with diabetes mellitus. The purpose of this paper is to report yet another relationship between plasma inorganic phosphorus and blood sugar which to our knowledge has not been described previously.

Procedure and methods. Phosphorus-glucose relationships were determined in 15 diabetic patients studied on the Metabolism Ward for prolonged periods. Ten patients had unstable diabetes; 4 were of the stable type(13). The group covered a wide range in age, duration of diabetes, and amount of exogenous insulin required. Throughout hospitalization, each patient received a chemically constant diet of identical foods and food values, the same menu being served every day. Data were obtained under optimal and suboptimal insulin regulation. Blood glucose and plasma or serum inorganic phosphorus were determined daily from the same sample of blood taken each morning before breakfast. Blood and urinary glucose were analyzed according to Somogyi's modification of the Shaffer-Hartman copper-iodometric titration method for "true" blood sugar(14). Plasma or serum inorganic phosphorus was determined either by modification of the method of Kitson and Mellon(15) or the method of Fiske and SubbaRow(16) and the urinary inorganic phosphorus by the method of Fiske and SubbaRow.

Results. A summary of the pertinent data is presented in Table I. It will be noted that there is a very wide range of blood glucose levels and, correspondingly, a fairly wide variation in plasma or serum inorganic phosphorus values. These 2 variables appeared to be related in a reciprocal manner, so that in general, low blood sugar levels tended to be associated with high plasma inorganic phosphorus levels and vice versa. In fact, a uniformly negative correlation between the 2 was found in all of the 15 patients studied (Table I). However, coefficients of correlation varied markedly from patient to patient. The degree of correlation did not appear to be dependent upon age, duration or type of diabetes, or amount of exogenous insulin required. Fig 1 illustrates the relationship between individual values for blood sugar and plasma inorganic phosphorus in a patient showing a fairly high coefficient of correlation.

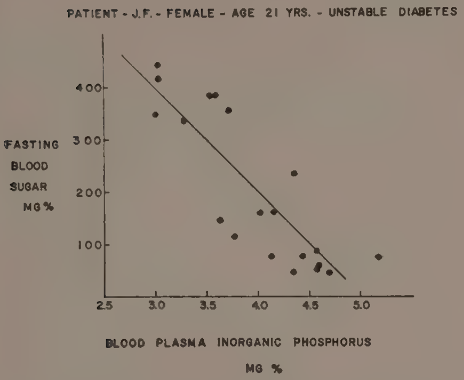

FIG. 1. Correlation between daily fasting blood sugar and plasma inorganic phosphorus in a patient with unstable diabetes mellitus.

In the one patient (E.M.) who was studied on 2 separate occasions, one year apart, the coefficients of correlation were found to be remarkably close, *i.e.*, –0.68 and –0.62 respectively. In contrast to the negative correlation between blood glucose and plasma inorganic phosphorus there appeared to be a slight tendency, if any, toward a low positive correlation between the 24-hour excretion of glucose and inorganic phosphorus in the urine.

Fig. 2 and 3 illustrate graphically the day-to-day levels of glucose and inorganic phosphorus in blood and urine and their interrelationships under different levels of insulin regulation in a patient with stable diabetes (M.W.) and in one with unstable diabetes (M.S.), respectively. It will be seen that in both patients, under optimal regulation, the spontaneous changes in blood sugar either above or below the normal base line appear to be associated with changes in plasma inorganic phosphorus in an inverse manner. On withdrawal of insulin (Fig. 2) or reduction of dosage to 50% or 60% of optimal (Fig. 3) the ensuing hyperglycemia is associated with a concomitant fall in plasma inorganic phosphorus. Note that the fall in inorganic phosphorus associated with the rise in fasting blood sugar in Patient M.W. occurred before the onset of the delayed glycosuria and was not associated with any appreciable change in the 24-hour excretion of inorganic phosphorus. Ketosis was absent in Patient M.W. In Patient M.S. ketonuria did not occur until several days of suboptimal insulin therapy had elapsed.

Discussion. The data presented demonstrate beyond all generally accepted statistical standards of reasonable doubt that, in the diabetics studied, fasting blood sugar and plasma phosphate level are negatively correlated. Whether or not this represents a causal relationship cannot be established by

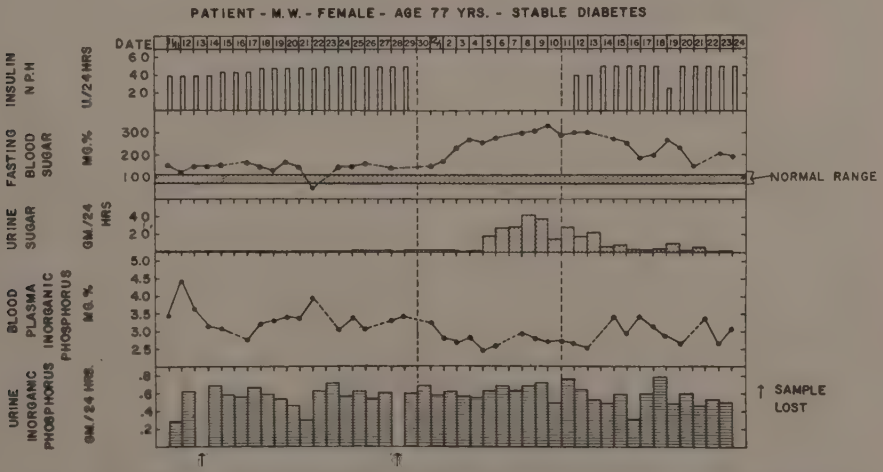

FIG. 2. Illustration of interrelationships of daily levels of glucose and inorganic phosphorus in blood and urine in a patient with diabetes mellitus under (a) optimal insulin regulation and (b) insulin withdrawal for an 11-day period.

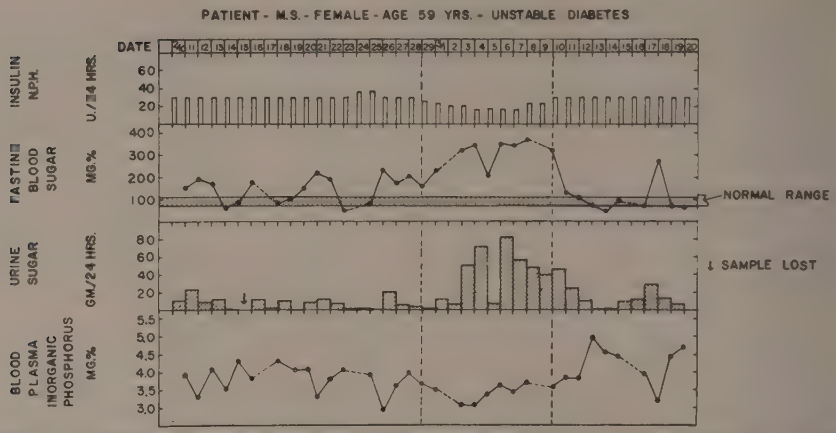

FIG. 3. Illustration of interrelationships of daily levels of inorganic phosphorus and daily levels of glucose in blood and urine in a patient with unstable diabetes under (a) optimal and (b) suboptimal insulin regulation.

this type of study. The consistency and reproducibility of the relationship in a given individual both with spontaneous changes in blood sugar and with changes induced by varying the dose of exogenous insulin would seem to favor a causal relationship. At any rate, the phenomena observed are of considerable interest since they are the reverse of those that had been anticipated in light of changes in serum phosphate with glucose utilization reported in short term experiments(2-12). If low blood sugar levels are taken as an index of increased utilization of glucose, one might expect low rather than high levels of phosphate. Conversely, decreased utilization of glucose as evidenced by hyperglycemia and glycosuria should lead to an accumulation of inorganic phosphorus in blood and urine. Indeed, elevation of both plasma and urinary phosphate does occur in diabetic acidosis; insulin therapy reduces these levels precipitously(17,18). In the present studies acidosis was absent. It should be pointed out that the present studies are not strictly analogous to the short term studies just alluded to. There is evidence that the short term change in phosphate caused by injection of glucose and insulin intravenously in diabetics is similar to that of normals(12). It is therefore possible that the phosphate-glucose relationships described reflect a different type of activity.

Can the observed phenomena be explained in part on a renal basis? Induction of hyperglycemia and glycosuria in normal animals (19) and man(20) has been shown to increase urinary excretion and clearance of phosphate while depressing tubular reabsorption of phosphate. In the present studies glycosuria does not appear to be an important factor in producing the phosphate changes since (1) the phosphate-glucose relationships occur in the absence of glycosuria and (2) phosphate and glucose excretion show at best only a very low positive correlation. This suggests that the observed phenomena reflect altered equilibria between phosphate stores and circulating phosphate rather than alterations in the renal mechanism governing phosphate excretion.

Summary. In a metabolic study of 15 patients with diabetes mellitus, a reciprocal relationship between fasting blood glucose and plasma or serum inorganic phosphorus was observed in all cases. In general, low levels of blood sugar tended to be associated with high levels of inorganic phosphorus and vice versa. Although a negative correlation was found in all patients, the coefficient of correlation varied markedly from patient to patient. There appeared to be a slight tendency, if any, toward a low positive correlation between the 24-hour excretion of glucose and inorganic phosphorus in the urine. The significance and possible mechanisms of the observed interrelationships are discussed.

We are indebted to Dr. S. Lee Crump, University of Rochester Medical Center, for statistical analyses in this paper.

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Reactions of Cyanocobalamin and Aquocobalamin with Proteins.[*] (22268)

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(Introduced by Earl A. Hewitt.)

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It now appears probable that absorption, transport, storage and function of cobalamins are accomplished by vitamin-protein complex formation. Although there is ample evidence that cobalamins are bound by proteinaceous substances present in gastric and duodenal secretions, blood serum, liver tissue, and milk there is relatively little information concerning the nature of bonds involved in the binding reactions. Cooley and co-workers(1) postulated that cobalamin-protein reactions may occur via cobalichrome formation analogous to the formation of ammonia and histidine cobalichromes. Van der Zant and Underkofler(2) presented evidence that suggests participation of both cobalt and substituted benzimidazole of cobalamins in cobalamin-gastric mucosal extract reaction. Absorption spectrum of purified cyanocobalamin-gastric protein complex, reported by Wijmenga and co-workers(3), indicates that cyano-group may still be present in the complex. Gregory and Holdsworth(4) reported the isolation of a similar cobalamin-protein complex from sow's milk and presented evidence indicating that the cyano-group is not displaced in the formation of this complex, and that the complex will not accept a second cyano-group. The latter authors made an extensive study of this binding reaction and concluded that tyrosyl residues of proteins but not sulfhydryl or amino groups, may participate. This investigation presents further

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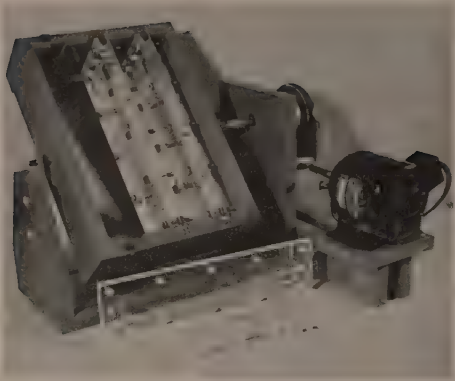

FIG. 1. Dialysis cells and rotation apparatus.

evidence concerning mode of linkage involved in cobalamin-protein reactions.

Dialysis method. Obvious inconsistencies in bacterial inhibition(5,6) and bacterial uptake(7) methods for differentiating free and bound cobalamins led to adoption of a dialytic procedure and radioisotope assay. Initial trials using dialysis bags and dialyzing to equilibrium or exhaustion, indicated that these methods were time-consuming and mechanically awkward. By standardizing the volume, membrane area and agitation a short-term or partial dialysis method was devised. Three identical Plexiglas dialysis cells were used. Each cell half contained a milled-out channel of about 30 ml capacity and a filling hole. The membrane, Visking dialysis tubing slit along one edge, was stretched between the two sections, separating and sealing the channels when cell halves were bolted together. Cells and rotation apparatus (3 r.p.m.) are shown in Fig. 1. In use, 25 ml of buffered protein-Co^{60}-labeled cobalamin mixture were dialyzed against equal volume of the same buffer for 4 hours in the dark at room temperature. Both solutions were then subjected to radio assay using a G-M liquid counting detector. Concentration of protein was adjusted to bind about half of the labeled cobalamin present. Using dialysis rate of free cobalamin, as determined by omitting protein from one of the 3 cells, the amount of cobalamin rendered non-dialyzable by a given amount of protein was calculated. Radioactive cobalamins were derived from a Co^{60}-labeled preparation (225 μc mg cobalamin) obtained from Merck and Co. on AEC allocation. Cyanocobalamin (vit. B_{12}) was prepared by treatment of an aqueous solution of this preparation with an excess of NaCN followed by nitrogen aeration in the dark at pH 6.5. Aquocobalamin (vit. B_{12b}) was prepared by simultaneous illumination and nitrogen aeration of an aqueous solution of this preparation at pH 5.5. All subsequent operations using these preparations were carried out under conditions of minimum light. Dialysis rate of vit. B_{12} followed Fick's diffusion law, that is, directly proportional to concentration differential across membrane at constant temperature and membrane area, and was unaffected by buffer type or concentration, or pH over the range 2 to 10. Dialysis rate of vit. B_{12b} was less straightforward, being complicated by adsorption by the cellophane membrane, and perhaps dimerization, the effect being more prominent in media of low ionic strength. By using 0.2 M phosphate buffer reproducible dialysis of vit. B_{12b} was obtained at observed rate approaching that of vit. B_{12}.

Results. Using the partial dialysis method, it was found that many proteins bind vit. B_{12b} to a much greater extent than vit. B_{12}, as indicated in Table I. Although not hitherto

TABLE I. Binding of Vit. B_{12} and B_{12b} by Proteins.

	Bound cobalamin, mμg/mg N†		
Protein preparation*	B_{12}, pH 6.6	B_{12b}, pH 6.6	B_{12b}, pH 4.0
Whole hemolysed blood	0	3.8	—
Hemolysed red cells	0	3.0	—
Plasma	0	5.0	.7
Plasma, heat denatured‡	0	5.8	—
Albumin (Armour)	0	3.0	.7
Lysozyme, 2× crystallized	0	0	—
Lysozyme, alkali denatured§	0	3.7	—
Ovalbumin	0	3.7	—
Trypsin, 2× crystallized	0	2.5	.5
Pepsin, " "	0	5.1	
Gastric mucosal extract	340	340	320

* Preparations contained 1 mg N/ml (0.013 mg N/ml for gastric mucosal extract) and 7.6 mμg cobalamin/ml in 0.2 M phosphate buffer. Blood preparations were of bovine origin.

† Sensitivity ca. 0.2 mμg cobalamin/mg N.

‡ Heated at 100°C for 5 min.

§ Incubated in 0.1 N NaOH at 37°C for 2 hr.

reported, this difference in extent of reaction between the two forms of vitamin can be expected as manifestation of cobalichrome formation. It was found that denaturation increased the vit. B_{12b} binding capacity of plasma and lysozyme. The binding capacity of 3 preparations was markedly decreased in acid media. Unreported spectrophotometric studies(8) indicated that vit. B_{12b}-histidine reaction is similarly depressed in acid media, as expected, and that histidine is the only amino acid giving evidence of cobalichrome formation in neutral solution. Similar compounds such as histamine, imidazole, carnosine, histidyl histidine and pyridine were found to form cobalichromes with vit. B_{12b}.

However, it cannot be inferred that benzimidazole is the only possible vit. B_{12b} binding site, for it was observed(8) that substances such as cellophane, filter paper, heparin, and nucleic acids (DNA and RNA) rendered nondialyzable considerable amounts of vit. B_{12b}. These substances did not bind measurable amounts of vit. B_{12}. The vit. B_{12b} bound by these substances and by the protein preparations was released by treatment with excess of cyanide in neutral solution.

The binding of as much as 5 mμg of vit. B_{12b}/mg of N by plasma is of interest for the physiological level is in the order of only 0.02 mμg of cobalamin/mg of N. Pitney, Beard and Van Loon have reported(9) that naturally occurring cobalamins are present in alpha and beta globulin components and that these components may also bind additional amounts of cobalamins, presumably vit. B_{12}, to a maximum of about 0.05 mμg/mg of N.

The position of bound labeled vit. B_{12} and B_{12b} in bovine serum was determined by subjecting to paper electrophoresis exhaustively dialyzed serum-cobalamin mixtures, and measuring radioactivity of the separate protein components(10). Serum used in this study was found to retain 4.3 mμg vit. B_{12b} or 0.092 mμg vit. B_{12}/mg of N, after exhaustive dialysis. Results presented in Table II indicate that all fractions of serum participated in binding of vit. B_{12b}, whereas only beta and gamma globulin components bound vit. B_{12}.

TABLE II. Position of Bound Co^{60}-Cobalamins in Bovine Serum Fractions Obtained by Paper Electrophoresis.

	Percent of activity			
Preparation	Albumin	α-	β-	γ-globulins
Serum + Co^{60}-B_{12b}	7	28	20	45
" " " -B_{12}	0	0	82	18

An entirely different situation was encountered in studying reaction of cobalamins with swine gastric mucosal extract. This extract consisted of water soluble, 50% alcohol insoluble, nondialyzable components of Ventriculin (Parke-Davis). As shown in Tables I and III, this preparation bound rather large and equal amounts of vit. B_{12} or B_{12b}, and binding of one form of vitamin was essentially blocked by prior excess of the other.

The effect of pH and excess cyanide on

TABLE III. Binding of Vit. B_{12} and B_{12b} by the Gastric Extract (GE).

Components*	Bound labeled cobalamin,† mμg/mg N
GE + Co^{60}-B_{12}	350
GE + excess B_{12b} + Co^{60}-B_{12}‡	0
GE + Co^{60} B_{12b}	350
GE + excess B_{12} + Co^{60}-B_{12b}‡	15

* Preparations contained 0.013 mg N/ml, 7.6 mμg labeled cobalamin/ml, and 2 μg nonlabeled cobalamin/ml, in 0.2 M phosphate buffer of pH 6.6.

† Sensitivity ca. 10 mμg cobalamin/mg N.

‡ Gastric extract and nonlabeled cobalamin allowed to react before adding labeled cobalamin.

TABLE IV. Effect of Hydrogen Ion Concentration and Excess Cyanide on Binding of Vitamin B_{12} by Gastric Extract.

	Bound vit. B_{12}, mμg/mg N	
pH*	No CN^-	Excess CN^-
2	320	—
4	320	—
6	330	—
6.6	340	340
8	320	—
10	330	250
10	340	250†
10	330	200‡
11	220	80
12	90	0

* Preparations contained 0.013 mg N/ml and 7.6 mμg Co^{60}-B_{12}/ml in 0.2 M phosphate buffer.

† Gastric extract and B_{12} allowed to react before adding cyanide. In all others, cyanide and B_{12} mixed before adding gastric extract.

‡ Contained 0.4 mg NaCN per ml. All others contained 0.1 mg NaCN/ml.

TABLE V. Fate of Cyano-Group of Vit. B_{12} in the Vit. B_{12}-Gastric Extract Reaction.

Treatment	Gastric extract	H_2O control
CN^- removed initially by N_2 aeration		
In dark, mμg	86	—
Illuminated, mμg	15	—
Idem	8	5
B_{12} added in dark, μg	4.5	4.5
(CN^- equivalent, mμg)	(87)	(87)
CN^- recovered by N_2 aeration		
In dark, mμg	14	12
Illuminated, mμg	79	83
B_{12} bound,* μg	3.4	—
(CN^- equivalent, mμg)	(66)	—

* Determined by the partial dialysis method.

binding of vit. B_{12} by this extract is shown in Table IV. The reaction was essentially unaffected by pH changes from pH 2 to 10 but was inhibited in more basic solution. Excess cyanide had no effect in neutral solution, but exerted a progressive inhibition above pH 10. The order of addition of excess cyanide and labeled vit. B_{12} to gastric extract did not affect the magnitude of inhibition. Excess cyanide did not affect the dialysis rate of free cobalamin. These data suggest that cobalamin is bound to this protein through the position sought by the second cyano-group.

That the cyano-group of vit. B_{12} is not displaced by the binding reaction is indicated by data presented in Table V. The cyanide method of Boxer and Rickards(11) was used. Gastric mucosal extract was first freed of cyanide by nitrogen aeration at pH 5. Labeled vit. B_{12} was then added and cyanide again determined, first after aeration in the dark, then while being illuminated. The amount of vit. B_{12} which had actually been bound by gastric extract was subsequently determined by partial dialysis. It appears that the first cyano-group is not displaced by the binding reaction, but remains in a photolabile position, presumably still with the cobalamin structure.

The effect of several treatments on vit. B_{12}-gastric mucosal extract reaction, as studied by partial dialysis, is shown in Table VI. The reaction was not affected by type or concentration of buffer. The effect of heat was similar to that reported by others using bacterial assay methods(2,12,13) and indicates that the complex is as heat-labile as the unreacted binding substance at pH 6.6. The report that cobalt-benzimidazole mixtures inhibit the reaction(2) could not be confirmed by this method. The suggestion that protein sulfhydryl groups may be involved in binding was shown to be unlikely, for the reaction was unaffected by excess para-chloromercuribenzoate. A cobalt porphyrin, reported to promote growth of vit. B_{12}-requiring microorganisms(14) was found to have no marked effect on the reaction.

Discussion. The marked reactivity of vit. B_{12b} suggests that suitable precautions should be taken against photoconversion when attempting to study vit. B_{12}-protein reactions. For example, heparin and nucleic acids were found to bind vit. B_{12b}, and not vit. B_{12} as had been reported(15). The binding or adsorption of vit. B_{12b}, by substances such as cellophane and filter paper point up difficulties involved in ascribing a physiological function to substances showing vit. B_{12b}-binding activity. Data presented in Tables I and

TABLE VI. Effect of Various Treatments on Binding of Vit. B_{12} by the Gastric Extract.

Treatment		Bound vit. B_{12}, mμg/mg N
Effect of buffer at pH 6.6		
No buffer		350
.1 M phosphate		320
.2 "		330
.4 "		330
.2 M acetate		340
Heating the gastric extract		
60°C, 1 hr		330
100°C, "		180
Heating the B_{12}-gastric complex		
60°C, 1 hr		330
100°C, "		180
Reagents	Molar excess*	
$CoCl_2$	2×10^4	320
Benzimidazole	3×10^3	330
" , $CoCl_2$	" , 2×10^4	320
" , " , heat†	" , "	270
p-Chloromercuribenzoate	1×10^4	340
Cobalt-hematoporphyrin	1×10^3	310

* Relative to original B_{12} concentration, 7.6 mμg per ml or 5.6×10^{-9} M.

† Heated at 100°C for 10 min. prior to addition of B_{12}. Gastric extract alone given same heat treatment bound 290 mμg B_{12}/mg N.

II suggest that binding of vit. B_{12b} by proteins, possibly via a cobalichrome linkage with histidine residues, is a rather general reaction involving little structural specificity of the proteins involved, and may therefore be of little physiological significance.

The limited study of the cobalamin-gastric mucosal extract reaction reported here is in general agreement with the more extensive study of Gregory and Holdsworth(4) who used ultrafiltration to study binding of vit. B_{12} by an isolated milk protein. However, there appears to be some discrepancy concerning the effect of excess cyanide on the reaction or reaction product. Gregory and Holdsworth observed no changes in absorption spectrum of cyanocobalamin-milk protein complex in presence of excess cyanide at pH values as high as pH 11. The data presented here indicate that dicyanocobalamin, in presence of excess cyanide, does not react completely with gastric extract, that excess cyanide dissociates pre-formed cyanocobalamin-gastric extract complex, and that these reactions are dependent upon cyanide concentration and pH. These results may represent a difference in nature of the binding reaction by the 2 preparations, or a difference in molar excess of cyanide used in the two situations. Although there have been reports (16,17) that cobalamin-peptide complexes are split by cyanide in neutral solution, the possibility exists that these are cobalichrome type structures and thus more susceptible to cyanolysis.

Summary. 1. A short-term dialysis procedure has been developed for studying cobalamin-protein reactions. 2. Aquocobalamin (vit. B_{12b}) was much more reactive than cyanocobalamin, being bound in rather large amounts by several protein preparations of diverse origin. In several instances binding was increased by denaturation and decreased in acid media. It is suggested that this type of binding may occur via cobalichrome formation. 3. A gastric mucosal extract bound large and equal amounts of aquocobalamin and cyanocobalamin, apparently at the same binding site. Excess cyanide has no effect on the cyanocobalamin-gastric extract reaction in neutral media, but exhibits an inhibiting effect at high pH levels. In this reaction the cyano-group of cyanocobalamin is not released, but remains in a photolabile position. It is suggested that the cobalt atom participates in the reaction and that it is the position occupied by the substituted benzimidazole moiety that is involved.

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Pituitary Stimulating Substance in Brain Blood of Hypophysectomized Rat Following Electric Shock "Stress".*† (22269)

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Release of ACTH following non-specific physiological stimuli is thought to involve the hypothalamus. However, the role of the hypothalamus in mobilization of ACTH is unclear. Following the ingenious experimental work of Harris and his co-workers (1-3), the concept of a hypothalamic humoral mediator in release of ACTH has gained wide support. The work of Harris *et al.*, Hume(4) and others(5-6), supports the thesis that areas in the posterior hypothalamus contain centers responsible for ACTH release. McCann and Sydnor(7) emphasize importance of anterior hypothalamus and suggest that pitressin, synthesized in this area(8), stimulates ACTH production following stress. The possible role of pitressin in stimulating release of ACTH has been emphasized by our work(9). However, Saffran *et al.*(10) reported that a contaminant of pitressin, but not pitressin itself, will cause ACTH release *in vitro*.

A minority of investigators, chiefly Vazquez-Lopez(11), believe that nerve fibers coursing in the hypothalamic-hypophyseal tract may serve to increase ACTH release by the anterior pituitary. To determine if a humoral substance is released from the hypothalamus following stress, the following experiment was devised: Venous brain blood of stressed hypophysectomized rat (donor animal) was administered into recipient animal in which endogenous mobilization of ACTH was impaired by electrolytic lesions placed in the hypothalamus (Fig. 1).

Methods. Hypophysectomy: Adult rats of the Addis-Slonaker strain were hypophysectomized and not used until at least 3 weeks later. The effect of ether anesthesia on eosinophils of several of these animals was determined. After each experiment, the sella was inspected with dissecting microscope for pituitary remnants. If found, the experiment was discarded. *Hypothalamic lesions*: Electrolytic lesions were placed in the hypothalamus by the Horsley-Clark stereotaxic apparatus as modified for the rat by Krieg(12). An indifferent electrode was placed in the rectum. The lesion electrode consisted of #21 gauge hollow tubing into which an entomological pin‡ was soldered at one end. The electrode was insulated with Formvar enamel§ except for 1 mm at tip of protruding pin. A current of 3 ma maintained for 15-20 seconds was used. Bilateral lesion sites were placed 4-7 mm anterior to intra-aural plane, 1 mm lateral and withdrawn 0.5-1 mm from base of skull. This location was generally within 1-2 mm of Bregma. Thirty to sixty % of the animals died within 2 days after the lesion. All lesioned rats surviving 3 weeks or more were subjected to ether anesthesia, found by Sydnor *et al.*(13) and Sayers *et al.*(14) to produce a stress reaction in rats as measured by increase in serum ACTH. Changes in level of circulating eosinophils were determined. Any rat having a decrease in eosinophils $>$ 20% was discarded as this indicated ability of lesioned rat to mobilize ACTH following stress was undisturbed. About 70% of lesioned rats were thus discarded. The remaining rats were used for experimental purposes. In addition, intact and adrenalectomized rats were exposed to ether and changes in eosinophils determined.

Experimental Procedure. Electric shock

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† This project was supported by grant from the U. S. Public Health Service and California Foundation for Medical Research.

‡ No. A-80; Size 1—Clay Adams Co., New York.

§ We wish to thank Mr. A. E. McCotter of Division of General Electric, Schenectady, for generously supplying Formvar enamel No. 9825 and thinner No. 9415.

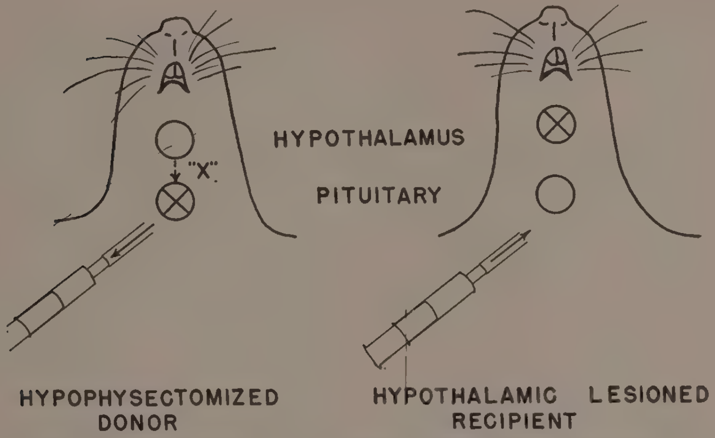

FIG. 1. Schematic representation of experimental design. The hypophysectomized rat is shocked with electricity and its venous brain blood collected in a syringe. This blood is injected intra-arterially into the hypothalamic lesioned rat. The dotted arrow represents a presumed secretion of the "Hypothalamic Hormone(s)" in response to the shock. Circles with × through them indicate removal of the designated areas.

stress was selected, for this could be controlled as to duration and intensity. An Ene-volt transformer¶ was employed. One lead was connected to each front foot and a current of approximately 24 volts administered at 3 per second for 2 minutes; 15 seconds on—5 seconds off. This constituted a severe stress, for the rats would struggle and convulse.

The experimental design is shown schematically in Fig. 1. At 9:00 A.M. a hypophysectomized donor rat was anesthetized with ether. The posterior facial vein (PF vein)** on one side was cannulated peripherally. The opposite PF vein was isolated and 0.2 cc heparin injected intravenously. The rat was maintained under light anesthesia for 60-90 minutes, then ether was withdrawn. Now the lesioned rat was anesthetized with ether and a blood sample immediately taken from free-flowing tail blood for eosinophil determinations. One carotid artery was cannulated peripherally; electric shock stress was then administered to the hypophysectomized donor rat. The isolated PF vein was ligated and about 2 cc of blood was withdrawn, during a 2-3 minute period, from the cannula in the contralateral PF vein. This blood was immediately injected into the lesioned recipient by means of the carotid cannula. Generally <15 minutes elapsed between anesthetizing the lesioned rat and injection of blood from the hypophysectomized rat. Eosinophils in the lesioned rat were then counted 30 minutes and 4 hours after injection of brain blood from the hypophysectomized rat. They were stained with propylene glycol-phloxine solution(15) and plated on a Fuchs-Rosenthal counting chamber. Four chambers were counted under low power. There was no evidence of blood incompatibility as determined by mixing 2 drops of blood from both donor and recipient animals.

Controls. Hypophysectomized rats were anesthetized with ether and the posterior facial vein was cannulated as described above. After 90 minutes, blood was withdrawn into the cannula and injected into the carotid artery of the same lesioned rats, which had received, or were subsequently to receive blood from "stressed"†† hypophysectomized rats.

¶ Correl & Correl, Chicago Heights, Ill.

** Dr. H. Hoagland(16), has shown that 70-80% of venous brain blood of the rat leaves the head via this vein.

†† Hypophysectomized rats shocked with electricity are hereafter referred to as "stressed" hypophysectomized rats.

TABLE I. Changes in Circulating Eosinophils in Rats 4 Hr after Exposure to Ether.

	(10) Normal	(8) Lesioned	(6) Hypophysectomized	(6) Adrenalectomized
% change in eosinophils	—58*	—11, —10, —20, —15, +13	+8, +43	—16
	—41	—20, —22	—15	— 3
	—35	+83	—25	+17
	—55	—19	—13, —15, +9	— 7
	—69	+18	—22	— 8
	—50	+16	— 8	—10
	43	+ 7		
	—53	+14		
	—38			
	—55			

* (—) % decrease; (+) % increase.

Injection of blood from "unstressed"‡‡ hypophysectomized rats occurred either a week before or after injection of brain blood from "stressed" hypophysectomized rats. Brain blood from "stressed" hypophysectomized rats was injected into adrenalectomized rats and hypophysectomized rats prepared as indicated above for the lesioned rat.

Results. Effect of ether stress on circulating eosinophils. Eosinophil changes occurring in 10 normal rats following exposure to ether are shown in Table I. A marked decrease occurred in each animal. This eosinopenia did not develop in 6 hypophysectomized rats exposed to ether, nor in 6 adrenalectomized rats similarly treated. This indicates that eosinopenia, following exposure to ether anesthesia, depends upon an intact pituitary-adrenal axis. In 8 lesioned rats anesthetized with ether eosinopenia also did not occur (Table I). Thus, it appears that a functional hypothalamic-pituitary-adrenal axis is necessary for the eosinopenic effect of ether. Several rats cited above were tested more than once for their response to ether.

Injection of brain blood from "stressed" and "unstressed" hypophysectomized rats into lesioned rats. The 8 lesioned rats which indicated no eosinopenia, following ether (Table I), were injected with brain blood from "stressed" and "unstressed" hypophysectomized rats. Changes in eosinophils are shown in Fig. 2. Lesioned rat #1 received blood from 2 different "stressed" hypophysectomized rats twice during 2 weeks, while the other lesioned rats received one injection of brain blood from a "stressed" hypophysectomized rat, and one injection of brain blood from an "unstressed" hypophysectomized rat. Thus, each rat served as its own control. The decrease in eosinophils in lesioned rats following injection of brain blood from "stressed" hypophysectomized rats was 65-90%. Following injection of brain blood from unstressed hypophysectomized rats into these same lesioned rats, the decrease was generally 25-35%; in one animal, eosinophils actually increased. In each case, regardless of whether the lesioned rat received "stressed" or "unstressed" blood, the 30 minute count was higher than the initial count.

Injection of brain blood from "stressed" hypophysectomized rats into adrenalectomized rats and hypophysectomized rats. This procedure was undertaken to determine whether eosinopenia following injection of brain blood from "stressed" hypophysectomized rats, was mediated by the adrenal-pituitary axis. In 4 adrenalectomized rats injection of brain blood from "stressed" hypophysectomized rats caused a decrease in eosinophils of only 15%, 1%, 19% and 11%. In 4 hypophysectomized rats into which brain blood from "stressed" hypophysectomized rats was injected, there was a decrease in eosinophils of 26%, + 27%, 23% and 4%.

Location of lesion sites. The brain was fixed in 10% formalin and several weeks later, sectioned through the hypothalamic area and stained in hematoxylin and eosin. Lesions were of massive nature, as intended, for we desired to block mobilization of ACTH without regard to minute hypothalamic areas. In 4 rats in which lesion sites were determined, the following areas were involved:§§ Rat #1 posterior hypothalamus including mammillary bodies, tuber cinereum and mammilothalamic tract. Rat #2 posterior hypothalamus including mammillary bodies, pitui-

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‡‡ Hypophysectomized rats not shocked with electricity are referred to as "unstressed" hypophysectomized rats.

§§ We wish to thank Dr. John D. Green for localizing the lesion sites.

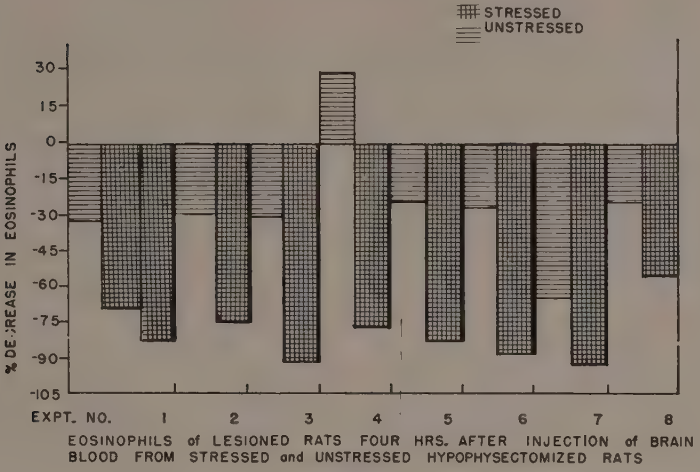

FIG. 2.

tary stalk, supraoptic and ventral hypothalamus. Rat #3 ventral hypothalamus including tuber cinereum; ventromedial nucleus and dorsomedial nucleus. Rat #4 posterior hypothalamus including tuber cinereum and mammillary bodies.

The location of massive lesions which blocked ACTH release, as measured by eosinopenia, were in the posterior and ventral hypothalamus and included mammillary bodies and tuber cinereum. McCann and Sydnor (7) observed that only anterior hypothalamic lesions, which destroyed the median eminence, blocked adrenal ascorbic acid depletion following laparotomy and subcutaneous epinephrine. Our lesion sites are in general agreement with Harris(1-3), Hume(4), Slusher & Roberts(6), and Porter(5), who similarly found areas in posterior hypothalamus responsible for ACTH mobilization. None of our lesioned rats demonstrated marked diabetes insipidus although daily water consumption records exceeded that of normal controls.

Discussion. Our data indicate that venous brain blood of "stressed" hypophysectomized rats will induce eosinopenia when injected into rats bearing hypothalamic lesions. This eosinopenia depends upon presence of intact pituitary-adrenal axis in recipient animal. Eosinopenia did not occur when brain blood from "unstressed" hypophysectomized rats was injected into these same lesioned rats. The assumption therefore appears justified that following "stress," brain blood of hypophysectomized animal contains a pituitary stimulating substance(s). These observations are compatible with the hypothesis of hypothalamic humoral control over ACTH secretion, and point to hypothalamus as the most probable site of hypophysiotropic principle demonstrated here. The demonstration that eosinopenia occurs only if pituitary and adrenal glands are present in recipient animal, further suggests that brain blood from "stressed" hypophysectomized rats exerts an eosinopenic effect via pituitary-adrenal axis, and has little, if any, direct effect upon level of circulating eosinophils.

The level of circulating eosinophils has been used by many workers investigating functional integrity of the hypothalamic-pituitary-adrenal axis(1,4,5). In some cases, however, eosinopenia may develop in adrenalectomized animals(17-18), following laparotomy and other severe stresses, although most investigators have found that this is not the case. We did not notice eosinopenia in adrenalectomized or hypophysectomized rats exposed to ether stress and minor neck surgery, nor did it occur in our rats with hypothalamic lesions. Thus under our conditions, eosinopenia depends upon intact hypothalamic-pituitary-adrenal axis.

Summary. Brain blood from "stressed" and "unstressed" hypophysectomized rats was injected into rats bearing hypothalamic lesions, and also into hypophysectomized and adrenalectomized rats. When brain blood from stressed hypophysectomized rats was injected into lesioned rats, a marked eosinopenia occurred; this did not occur when unstressed brain blood was injected into those same lesioned rats. This eosinopenia depended upon the presence of an intact pituitary-adrenal axis.

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Effects of Vitamin E, N,N[1]-Diphenyl-Para-Phenylene Diamine, and Fish Liver Oil on Reproduction in Turkeys.*† (22270)

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A deficiency of vit. E has recently been reported in turkey breeder hens fed practical rations(1,2). The lenses of the eyes of embryos from the eggs laid by hens fed diets deficient in vit. E were cloudy in the central portion(3). In vit. E studies with chicks, Singsen *et al.*(4) found that the unsaturated fat level of the diet markedly influenced the development of encephalomalacia and that the antioxidant N, N^1 diphenyl-para-phenylene diamine (DPPD) was about as effective as vit. E in preventing the disease. The effects of DPPD, fish liver oil and alpha tocopheryl acetate on the reproductive performance of turkeys are reported herein.

Procedure. Broad Breasted Bronze turkey hens were distributed into 12 pens of 11 hens each. These birds had been fed a practical diet during the growing period and were reared on range. A basal diet of the following percentage composition was used: 66 ground corn, 18 soybean oil meal, 2.5 dehydrated alfalfa, 3 herring fish meal, 3 dried whey, 3.7 limestone, 3.5 steamed bone meal, 0.3 iodized salt, 0.0125 manganese sulfate and a vitamin mix supplying 680 I.C.U. vit. D, 1135 I.U. vit. A, 1 mg riboflavin, 6.2 mg pantothenic acid and 2.5 mg vit. B_{12} per lb of

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† Scientific Paper No. 1463 Washington Agric. Exper. Stations, Pullman. Project No. 715.

TABLE I. Effect of Vit. E, Fish Liver Oil and DPPD on Reproductive Performance of Broad Breasted Bronze Turkeys and upon Tocopherol Content of the Yolks.

Supplement to basal	Pen No.	% fertility	% embryonic mortality		Pips	% hatch. fertile eggs	Total tocopherol, μg/g yolk
			1-24 days	25-28 days			
None	33	56.9	7.8	14.3	7.4	70.6	32.2
	39	75.7	8.7	12.7	12.7	62.2	31.3
	Avg	66.5	8.3	13.4	10.3	66.0	31.8
Vit. E	36	85.2	4.7	8.0	13.7	74.6	74.2
	41	78.5	5.8	7.9	7.2	79.5	71.2
	Avg	81.8	5.2	7.9	9.9	77.1	72.7
Pollack liver oil (PLO)	35	67.2	15.3	44.5	9.6	30.6	17.1
	37	52.2	7.1	36.3	8.8	47.8	17.8
	Avg	58.6	11.5	41.2	9.2	38.6	17.4
PLO + Vit. E	34	75.8	9.8	7.5	5.6	77.0	27.1
	42	80.8	5.4	9.0	9.7	76.0	16.0
	Avg	77.9	7.7	8.2	7.5	76.5	23.0
PLO + DPPD	31	73.7	7.3	13.7	7.7	71.4	16.5
	38	72.8	3.3	13.3	7.2	76.3	19.7
	Avg	73.2	5.1	13.5	7.4	74.0	18.0
PLO + DPPD + Vit. E	32	68.2	3.9	7.9	6.2	82.0	24.6
	40	70.2	9.3	6.9	5.8	78.0	23.7
	Avg	69.2	7.2	7.2	6.0	79.5	24.3

feed. Duplicate lots were fed the basal diet and the basal diet supplemented with 20 I. U./lb alpha tocopheryl acetate, 2% pollack liver oil, 2% pollack liver oil plus 20 I.U./lb alpha tocopheryl acetate, 2% pollack liver oil plus 0.025% DPPD and 2% pollack liver oil plus 20 I.U./lb alpha tocopheryl acetate. During the first 9 weeks of the experiment, Broad Breasted Bronze toms were maintained in a separate pen. Semen was collected from these toms and pooled for artificial insemination of the hens. During the last 12 weeks of the experiment, one tom was placed in each pen and the toms were rotated weekly. All eggs laid were stored at approximately 55°F and incubated every 2 weeks. The eggs were candled on the 7th and 24th day of incubation to determine infertile eggs and eggs containing dead embryos. The experiment was conducted for a period of 21 weeks. During the latter part of the experiment, a sample of 6 eggs from each pen was analyzed for total tocopherol content.

Results. The results of this experiment are presented in Table I. The data on fertility are based only on eggs laid by the hens after they were naturally mated. These results suggest that natural diets such as used may be deficient in vit. E to the extent of causing reduced fertility of eggs. The addition of alpha tocopherol acetate to both the unsupplemented basal diet and to the basal diet containing 2% fish liver oil increased the percentage of fertile eggs. In results with turkeys reported by other investigators(1,2) fertility was not affected by a vit. E deficiency. Singsen *et al.*(5), however, obtained evidence suggesting an improvement in fertility in chickens by addition of vit. E to the breeder ration. It should be mentioned that fertility in the experiment being reported was determined only by candling. It is possible that some of the eggs classified as infertile may have contained embryos that died at a very early stage.

Addition of vit. E to the basal diet increased hatchability of fertile eggs. When 2% fish oil was added to the basal diet, hatchability of fertile eggs decreased from 66 to 39%. The addition of vit. E or DPPD or a combination of the two in the presence of fish oil markedly improved hatchability. These results show that the presence of an unsaturated oil in the diet greatly accentuated the deficiency of vit. E. The results also show that both vit. E and DPPD act to counteract the deleterious effect of unsaturated oil on hatchability. An examination of the data on time of embryonic mortality shows that there was very little difference in the percentage of embryos dying from 1 to 24 days among the various treatments. In the case of embryos dying during the 25-to-28-day period, however, there was a marked difference. When fish liver oil was added to the basal diet embryonic mortality during this period greatly increased. In all cases where vit. E was added to the diets embryonic mortality during this period averaged about 8%. In the unsupplemented basal treatment and in the treatment with fish oil and DPPD alone, mortality during this period averaged about 13.5%. In previous reports by Jensen (1) and Atkinson *et al.*(2), the major difference between vit. E deficient and vit. E supplemented lots was in the embryonic mortality from the 25th to the 28th day of incubation. The fact that DPPD alone did not reduce the embryonic mortality during this period to a level equivalent to vit. E supplementation would suggest that the antioxidant was not completely effective in bringing about maximum hatchability. This may have been due to an inadequate level of the antioxidant. It is also possible that the antioxidant was only capable of protecting the vit. E naturally present in the basal diet. If this is the case, one would not expect the results with the antioxidant to be any better than those with the unsupplemented basal diet.

The analysis of egg yolks from the various treatments showed that alpha tocopheryl acetate added to the unsupplemented basal diet gave a marked increase in total tocopherol content of yolks of eggs laid by these hens. The addition of pollack liver oil to the basal diet caused a marked reduction in tocopherol content of the yolks. The addition of vit. E, DPPD, or a combination of the two with fish liver oil slightly increased the tocopherol content of the yolks, but did not bring it back to the level of the unsupplemented basal diet. This was surprising, since the hatchability of these eggs from hens supplemented with vit. E was as high as eggs from hens fed the basal diet plus vit. E without fish oil and was higher than eggs from hens fed the unsupplemented basal diet. The results suggest that vit. E and DPPD may be protecting some other unknown nutrient or metabolite which is essential for reproduction in the turkey. Bunnell *et al.*(6) proposed that the protective action of DPPD against encephalomalacia in chicks might be due to a stimulating effect on the metabolism of the unsaturated fats rather than a purely vit. E protective action.

Summary. Fish liver oil added to the diet greatly accentuated a deficiency of vit. E in turkey breeders. A reduction in fertility due to a vit. E deficiency was suggested. Both alpha tocopheryl acetate and DPPD counteracted the deleterious effect of fish oil on hatchability. With fish oil in the diet, tocopherol levels of the yolks of eggs were not appreciably increased by either vit. E or DPPD even though hatchability was near maximum.

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Cultivation of Human Epithelial Cells in Tissue Culture.* (22271)

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(With technical assistance of S. Robert Wilson.)
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Several investigators have studied serial propagation of normal cells in tissue culture, particularly in the last 3 years, partly because of need for a substitute for fresh monkey kidney cells in mass production of poliomyelitis vaccine. Many other uses can be envisaged. Chang(1,2) accomplished this and 2 cell lines established by him from presumably normal human tissues were received in this laboratory early in 1955. This communication describes our experience in serial subcultivation of those 2 cell lines to determine their adaptability to various laboratory technics or use in diagnostic and investigative studies. Data are presented on media requirements, growth in various culture vessels, preservation at reduced temperatures, lyophilization, adaptation to growth in nonhuman sera, and treatment of contaminated cultures. The use of these cells for propagation of various viruses will be presented later.

Materials. Cell lines. Human conjunctiva had been originally removed from the bulbar conjunctiva of a child at strabismus operation, Feb. 17, 1954. The tissue was then considered nonmalignant. When received in this laboratory Feb. 3, 1955 it was in the 37th serial tissue culture passage. The human kidney had been removed from a hydrocephalic child at the time of subarachnoid ureteral anastomosis on 5-12-54. The kidney was then considered normal on gross examination. When received in this laboratory on 2-3-54, the cell line was in the 25th serial tissue culture passage. *Media.* Human, horse, calf, monkey, rabbit, and chicken sera were used in these experiments. Solution 199 (3) and Hank's balanced salt solution were prepared in the usual way. Eagle's medium (EM)(4) was prepared in 1x concentration and neutralized with 4.4% sodium bicarbonate prior to use. Soybean trypsin inhibitor was prepared in a 1% stock solution in Hank's balanced salt solution. Trypsin (Difco 1:250) was prepared in a 0.125% solution in phosphate buffer solution (pH 7.6). The above solutions were sterilized by sintered glass filtration. Chick embryo extract was prepared fresh weekly from 9 day viable embryos. The standard medium for cultivation of cells was Chang's basic medium (CM), *i.e.*, 20% human serum, 5% chick embryo extract, 75% balanced salt solution, and 0.001% crystalline soybean trypsin inhibitor. Antibiotics were added to a final concentration of 100 units per ml of penicillin, 100 μg of streptomycin, and 25 μg of nystatin.

Results. The 2 cell strains received were cultivated in 3 basic media to which were added varying percentages of human or animal sera. No difficulty was encountered in growing these cells. To evaluate quantitatively ability of these media and sera to support proliferation of the 2 cell strains, the cell count method described by investigators (2,5-8) was modified to fit our needs, *viz.*, preparation of standard cell suspension for inoculation by trypsinizing the cells growing in a milk dilution bottle culture. Total viable cells were enumerated by inclusion of 1:50000 neutral red in the trypsin, dispersing cells by gentle agitation and counting in a hemocytometer. Trypsin was removed after centrifugation at 1000 rpm for 10 minutes in International No. 1 centrifuge. The sedimented cells were resuspended in balanced salt solution and homogenously dispersed by a magnetic stirrer. Replicate roller tubes were inoculated with equal number of cells contained in 0.25 ml of suspension using automatic syringe and 4 tube cultures were fed with each medium. Replicate cultures were incubated at 37°C and refed with the same medium after 4 days. After 7 days incubation cells from replicate tubes were removed by trypsin containing 1:50000 neutral red,

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TABLE I. Effect of Animal Sera on Proliferation of Human Conjunctiva Cells Cultivated in Chang's Medium plus 20% Serum.

Serum	Avg No. cells per tube × 10^3	Proliferation index (7 days)
Horse 1	0	0
2	0†	0
3	49	7.4
4	41	6.2
Calf 1	47	7.1
Rabbit 1	6.7	1.0
2	17.2	2.6
Sheep 1	14	2.1
Chick	21	3.1
Control (human #20)	77	11.6

* Inoculum/tube = 6.6 × 10^3 cells of Passage C_{55}.

† 0 indicates that all original cells inoculated into tubes were degenerated or lysed.

pooled, dispersed, and enumerated. Two separate cell counts were done for each pool of 4 cultures. A standard human serum was included in each test for comparison. Human sera from 38 separate donors were employed. Because of Chang's early experience (1) that 1 out of 5 human sera were toxic for conjunctiva cells and 2 out of 5 supported multiplication slowly, great pains were taken by us to collect these various sera carefully and in large quantity so that if a good serum was obtained enough would be on hand to carry the cells for a number of passages.

Only 2 of the 38 sera proved to be toxic for the tissue culture cells; both were toxic to both conjunctiva and kidney cells. Toxicity consisted of rounding and clumping of cells. All other sera were non-toxic and maintained both cell strains in normal morphologic state of clear epithelial-like sheets.

Sera from different donors varied greatly in ability to support cellular multiplication. The proliferation index (number of cells at end of 7 days ÷ number of cells inoculated) varied from 5.4 to 9 for conjunctiva and from 2.3 to 18.2 for kidney cells when different human sera were employed in Chang's medium. Sera from various animals varied in ability to support multiplication of conjunctiva cells when substituted for human sera in CM (Table I). Horse and calf sera seemed superior to rabbit, sheep, and chick sera tested but were not as good as human serum control. Two of the 4 horse sera tested were toxic.

Multiplication of conjunctiva and kidney cells when cultivated in CM, EM, and 199 media with added human, horse, or calf serum in 10 and 20% concentrations is shown in Tables II and III. These data indicate that both cell types multiplied faster in media with 20% serum than with 10% serum. Conjunctiva cells multiplied fastest in solution 199, CM and EM in that order. EM with human serum supported good growth but with horse or calf serum it was clearly inferior. Kidney cells responded in a similar manner. Beginning June 1955 efforts were made to adapt both cell lines to media containing horse and calf serum and no human serum. The adaptation was easily accomplished and conjunctiva have been carried since 6-24-55 for 18 serial passages from C_{50} to C_{68} in both 20% horse or calf serum in EM and 199 solution. Kidney cells have been serially subcultivated

TABLE II. Effect of Serum Concentration on Proliferation of Human Conjunctiva Cells Cultivated in Chang's, Eagle's, or 199 Medium with Added Human, Horse or Calf Serum.

Media	Avg No. cells per tube × 10^3 10% serum	20% serum	Proliferation index (7 days) 10%	20%
Chang's + human serum (21)	42*	54	7.0	9.
Eagle's human (21)	31	55	5.1	9.
" horse	10	26	1.6	4.3
" calf	12	11	2.0	1.8
199 horse	16	66	2.6	11.
199 calf	27	66	4.5	11.

* Inoculum/tube = 6 × 10^3 cells of Passage C_{56}.

TABLE III. Effect of Serum Concentration on Proliferation of Human Kidney Cells Cultivated in Chang's, Eagle's, or 199 Medium with Added Human, Horse or Calf Serum.

Media	Avg No. cells per tube × 10^3 10% serum	20% serum	Proliferation index (7 days) 10%	20%
Chang's + human serum (23)	13*	60	2.6	12
Eagle's human (23)	7	16	1.4	3.2
" horse	1.4	25	.28	5.0
" calf	1.1	4.4	.22	.88
199 horse	20	42	4.0	8.4
199 calf	24	64	4.8	12.8

* Inoculum/tube = 5 × 10^3 cells of Passage K[illegible].

TABLE IV. Total Cell Yield in Various Culture Vessels Inoculated with Either Human Conjunctiva or Kidney Cells.

Vessel (vol, ml)	Vol of media (ml)	Range of cell population after 7 days cultivation (human or horse serum) (× 1000)
Roller tube (18)	1	26 to 182
Milk dilution bottle (230)	10	250 to 3150
Blake bottle (1000)	50	1600 to 5800
Povitsky bottle (5000)	250-500	10000 to 30733

in these 4 media since 7-15-55 for 14 serial passages from K_{41} to K_{55}.

The 2 cell lines have been cultivated in microscopic glass chambers and hanging drop preparation for direct microscopy; roller tubes, milk dilution bottles, Blake bottles, and Povitsky bottles. In each type of culture vessel the range of cell population after approximately 7 days cultivation is shown in Table IV. Both types of tissue may be maintained in good morphologic condition for 2-3 weeks at 37°C by refeeding cultures twice weekly. For maintenance of conjunctiva cell cultures at a reduced metabolic rate they have been stored at 32°C, at 5°C, and at –70°C. Kidney cell cultures have been maintained at 32°C and 5°C. At 32° C cultures were stored with the usual volume of nutrient media and refed once weekly. At this temperature cultures maintained their usual morphologic state for 4-5 weeks. At 5°C they were stored with only a thin layer of nutrient media for 2 months. Conjunctiva roller tube cultures were quick frozen in acetone-CO_2 bath after removal of supernatant fluid, stored 2 days and revived upon feeding and incubation at 37°C. Eleven such conjunctiva tubes were lyophilized and stored at room temperature for one day. When they were refed and incubated at 37°C for 5 weeks with medium change once a week, 3 of the 11 cultures revived and formed sheets of new cells.

Centrifugally sedimented conjunctiva cells without added fluid were alternately quick frozen and thawed 10 times. When tested for viability, 1 of 4 cultures grew a sheet of cells in 7 days. When kidney cells were suspended in EM plus 20% horse serum in concentration of 1000000 cells/ml and then frozen and thawed 10 times, no viable cells were noted upon subsequent cultivation.

Non-viable cell extracts of both conjunctiva and kidney cells were prepared by this latter method. These were each injected into monkey kidney tissue cultures, 10 embryonated eggs, 4 rabbits, 4 guinea pigs and 10 mice to note presence of herpes virus simiae, lymphocytic choriomeningitis virus, tubercle bacilli, bacteria or any orphan virus which would manifest itself in these tests. No agents were found in either cell line by any of these tests.

Contamination of a batch of monkey tissue cultures is disappointing but economically not too serious because they can be discarded and duplicated within a week by setting up new tubes with fresh monkey kidney. However, bacterial or mold contamination is a severe threat to an established strain of cells which may have taken months to establish in serial tissue culture. To overcome this, we have applied the principles used in clinical medicine. The tissue culture fluid is streaked on agar plates and antibiotic discs added. The antibiotic of choice is added to tissue culture medium for several passages. Cultures contaminated with molds or yeasts have been successfully treated by adding 50 μg of nystatin for several passages.

To note the virus yield from poliomyelitis stool specimens inoculated into tissue cultures of conjunctiva cells as compared to tissue cultures of monkey kidney cells, 2 stool specimens from poliomyelitis patients were inoculated into 3 roller tube cultures each of conjunctiva and monkey kidney. Cytopathogenicity occurred after 1 day in monkey kidney cultures and after 2 days in conjunctiva cultures. After 5 days, supernates from each cell type were pooled and typed with specific antisera and titrated in monkey kidney cells to determine the virus titer in the fluids. The poliovirus isolated was Type I in stool A and the virus titer in the 5-day supernate from monkey kidney cells was $10^{6.33}$ per ml as compared to $10^{6.41}$ per ml from conjunc-

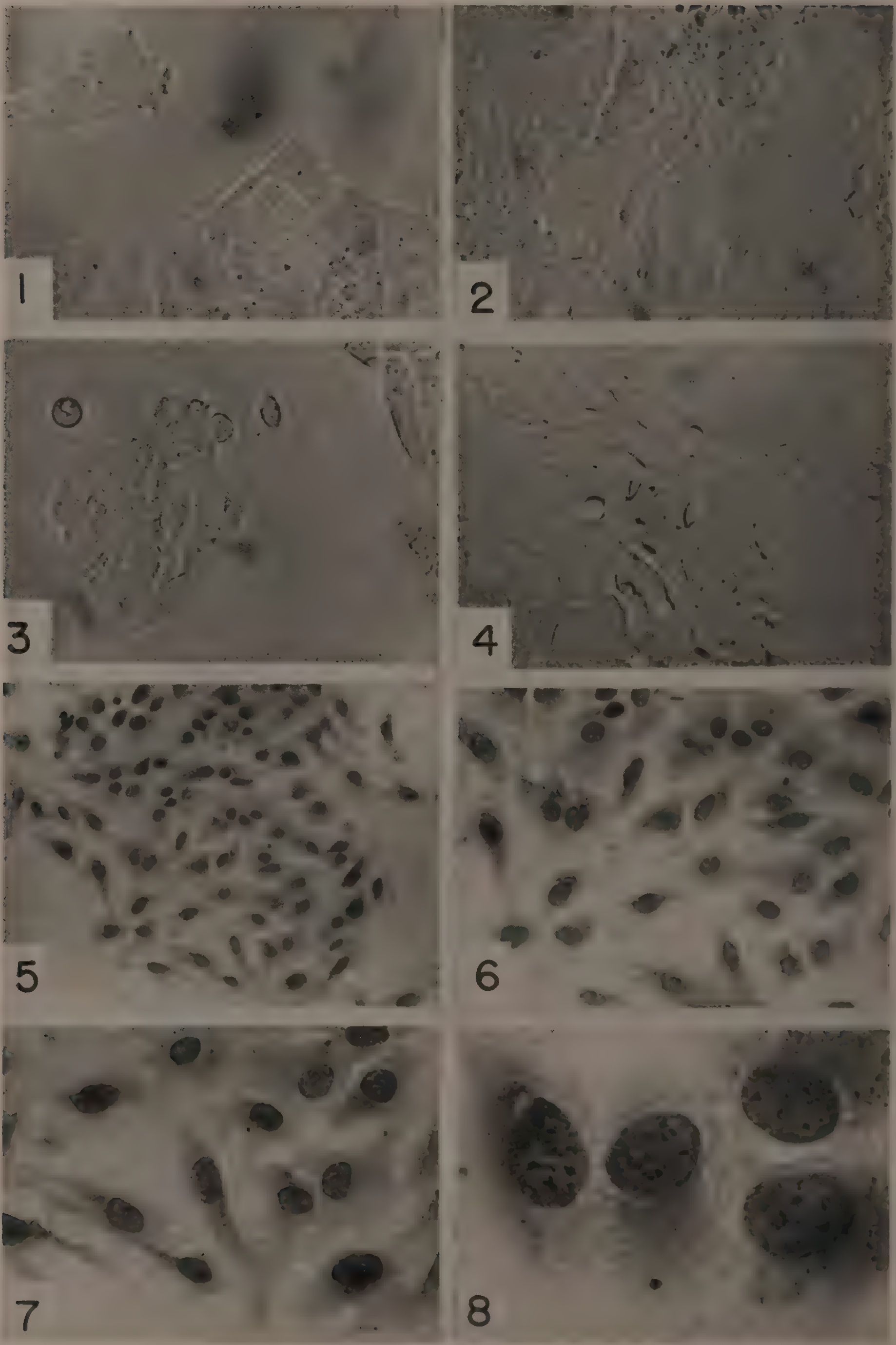

Plate 1

FIG. 1. Conjunctiva cells, 2 days after subcultivation showing islands of flat monolayer epithelial cells. Unstained. × 220.

FIG. 2. Conjunctiva cells, 10 days after subcultivation showing thick sheet of cells with some clumping. Unstained. × 190.

FIG. 3. Kidney cells, 2 days after subcultivation showing islands of cells. Unstained. × 220.

FIG. 4. Kidney cells, 7 days after subcultivation showing thick sheet of cells. Individual cell borders are indistinct because of light diffraction by underlying cells. Unstained. × 190.

FIG. 5-8. Conjunctiva cells, 2 days after subcultivation, showing cellular and nuclear detail. Magnification × 100, 250, 400, and 1000, respectively. Osmic acid fixation, toluidine blue stain.

tiva cells. The virus isolated was Type II in stool B and the virus titer in the 5th day supernate from monkey kidney cells was $10^{6.71}$ per ml as compared to $10^{6.76}$ per ml from conjunctiva cells.

Histology. The morphologic appearance of these 2 cell lines is shown in Plate 1. Both produce large epithelial-like cells with a nuclear cytoplasmic ratio of about 1-6. The cells fit together in flat monolayer sheets in young cultures but form thick sheets, sworls, and thick clumps in older cultures. There is remarkable uniformity of cells although occasional abnormal mitotic figures are observed and rarely a binucleate cell. Polyploidy has been observed but its significance cannot be interpreted in the present state of knowledge of how cells behave in tissue culture. It is of interest that the microscopic appearance of these cells has not changed since the original publication by Chang(1).

Discussion. It is believed that these cell lines established by Chang represent the first human cells derived from normal tissues and successfully cultivated serially *in vitro* over a prolonged period of time. As such, they offer a research tool of great potential usefulness in study of virus diseases and other problems.

These cells can be grown in a variety of containers for microscopic studies or for mass production or chemical analysis. They thrive in media free from human serum and in relatively simple synthetic media. The toxicity of many human sera for these cells, observed by Chang during earlier subcultures, has not been observed by us, nor has it been observed by Chang in recent months. Presumably this is a reflection of a change in the cells, the nature of which is still quite obscure. The cells can be stored for prolonged periods at low temperature and some will survive freezing and lyophilization. Tests for presence of a resident virus in these cultures have been negative, and sublines have been maintained for many generations without any human serum added. This was done to prevent contaminating cultures with a hepatitis virus or other human pathogenic agent(9).

In very limited studies one of the cell lines appeared to be as sensitive as monkey kidney cells for the isolation of poliomyelitis viruses. If this proves to be true, they might be very useful for laboratory diagnosis. This is now being tested. Because of the ease of handling these cells and because they are of human origin, they should be useful in the search for a laboratory method to grow viruses which as yet have not been cultivated in a practical manner such as measles and German measles. Other diseases such as infectious mononucleosis, infectious polyneuritis, mesenteric adenitis and hepatitis may be explored with such cell lines.

Since the conjunctiva and kidney cells grow so well in tissue culture, one might ask whether they have also acquired the power of independent growth *in vivo*. Studies are under way using the technics of Greene(10) of implantation in the brain and anterior chamber of guinea pigs, and the technics of Toolan(11-14) and Moore(15) of inoculation of irradiated rats.

Summary. 1. Experience is described in the serial subcultivation of 2 human cell lines derived from conjunctiva and kidney and established in serial passage *in vitro* by Chang. 2. Thirty-eight human sera were compared in their ability to support multiplication of the 2 cell lines. Two of the sera were toxic to both cell lines. The remaining 36 sera were non-toxic but varied in their ability to support cellular multiplication. Horse and calf

sera were superior to rabbit, sheep, and chicken sera but inferior to the human serum control. 3. Comparison was made of the multiplication of the 2 cell lines when cultivated in Chang's, Eagle's, and 199 media with added human, horse, or calf serum in 10 and 20% concentration. 4. Conjunctiva cells have been serially subcultivated for 18 passages in media containing horse or calf serum but no human serum. Kidney cells have been carried for 14 passages in such media. 5. Conjunctiva and kidney cells survived for varying periods when stored at 32°C or 5°C. Some sedimented conjunctiva cells survived 10 freezing-thawing cycles and formed a sheet of cells when incubated at 37°C. 6. Three of 11 conjunctiva cultures survived lyophilization and revived when reincubated at 37°C for 5 weeks. No contaminating bacterium or virus could be detected in these cell lines by the injection of nonviable cell extracts into tissue cultures of monkey kidney, embryonated eggs, rabbits, guinea pigs, and mice. 7. A method is described for the treatment of contaminated tissue cultures. 8. The conjunctiva cells gave results comparable to monkey kidney cells when used for isolation of poliomyelitis virus from 2 stool specimens.

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Serial Biochemistry of Serum and Cerebrospinal Fluid in Amaurotic Family Idiocy (Tay-Sachs Disease).* (22272)

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Studies in Amaurotic Family Idiocy (AFI) have thus far been principally concerned with histologic examination and biochemical analysis of brain tissue. These latter investigations resulted in isolation of a new cerebroside-like substance called a "ganglioside" and differing from other cerebrosides by the presence of neuraminic acid(1-3). In contrast to other lipoidoses, it is generally assumed that there are no characteristic serum changes in AFI.

The present report is concerned with serial studies of the neuraminic acid, protein and lipid fractions in serum, and cerebrospinal fluid (CSF) proteins in a group of 7 children with infantile AFI, the diagnoses in most instances confirmed at autopsy. Similar biochemical studies of 2 children with Schilder's disease and gargoylism, serving as controls, were also carried out. Most children in this survey were admitted to this institution shortly after clinical inception of the disease and remained hospitalized until after death. The increased longevity of some patients with AFI in this study(4), afforded greater opportunity for carrying out such serial biochemical

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TABLE I. Serum and C.S.F. Protein Data in Amaurotic Family Idiocy and Related Disorders.

Serum electrophoretic data

Diagnosis	T.P., g %	Alb., g %	Alpha-1, g %	Alpha-2, g %	Beta, g %	Gamma, g %	A/G, %
Tay-Sachs disease, 7 p, 20 d*	6.80 ± .35† <.01‡	3.70 ± .33 <.01	.50 ± .09 .40	.98 ± .17 <.01	.96 ± .13 —	.65 ± .21 <.01	1.20 ± 2.10 .15
Schilder's disease, 1 p, 3 d	7.39§	3.50	.59	.95	1.28	1.08	.90
Gargoylism, 1 p, 4 d	7.12§	3.57	.52	.95	1.25	.83	1.00
Normal subjects	7.26 ± .39	4.07 ± .29	.48 ± .09	.66 ± .11	.96 ± .20	1.01 ± .20	1.30 ± .18

C.S.F. proteins (chemical)

Diagnosis	T.P., mg %	Alpha, mg %	Alpha/T.P., %	Gamma, mg %	G.G./T.P., %	Serum neuraminic acid, mg %
Tay-Sachs disease, 7 p, 20 d*	41.7 ± 11.32† .10‡	5.9 ± .94 <.01	16.2 ± 3.11 .04§	1.7 ± .98 <.01	4.9 ± 2.88 <.01	89.6 ± 7.10 <.01
Schilder's disease, 1 p, 3 d	51.6§	7.9	15.2	9.5	21.3	82.7
Gargoylism, 1 p, 4 d	50.8§	9.5	18.7	3.4	6.6	84.4
Normal subjects	37.5 ± 5.0	8.6 ± 1.2	18.2 ± 2.2	4.0 ± 1.0	9.4 ± 1.8	72.9 ± 7.4

* p = patients; d = determinations. † Mean ± S.D. ‡ P. § Mean.

analyses.

Methods. (a) *Serum:* The procedure used for the electrophoretic protein determinations has been described(5,6). Total proteins were determined by biuret procedure(7) with frequent checks against the micro-Kjeldahl method. Total lipids were determined gravimetrically with the Bloor(8) method; phospholipids with the modified colorimetric procedure of Youngburg and Youngburg(9) and free fatty acids by the titrimetric technic of Kaiser and Kagan(10). Total and free cholesterol values were obtained with colorimetric method of Zak, *et al.*(11) and cholesterol esters and cholesterol ester/total cholesterol ratios were calculated from these experimental results. Neutral fats were calculated from the above data by the formula given in the article by Goldbloom(12). Lipase values were determined by the method of Goldstein, *et al.*(13) and neuraminic acid by a modification of the procedure described by Winzler (14). (b) *Cerebrospinal Fluid*: Total proteins were determined by a modification of the Weichselbaum biuret method(15). Gamma globulins were determined by quantitative protein flocculation-ninhydrin colorimetric procedure(16) and alpha globulins with a newly developed turbidimetric method(17) as modified from a previously described technic of Jacox(18) for serum alpha globulins. The simultaneous serial studies on both serum and CSF were generally carried out in duplicate at bi-monthly intervals for periods to 10 months. While the derived biochemical data from the AFI cases were *statistically* analysed (Tables I, II) it was deemed unnecessary to apply such an evaluation to the two control cases because of insufficient number of determinations.

Results. Previous experience with other neurological entities of a degenerative nature such as multiple sclerosis, Schilder's disease, amyotrophic lateral sclerosis, etc., has shown consistent alterations of both the serum and CSF protein fractions as determined by electrophoretic and biochemical technics. The significance of these changes is not related to alterations in the individual protein fractions, but rather in the entire "protein profile"(19,20). For example, while there is a notable increase in CSF gamma globulin in multiple sclerosis, no corresponding rise in serum gamma globulin is evident, in contrast to many infectious types of disease, *e.g.*, neurosyphilis, wherein both CSF and serum gamma globulin fractions rise above the anticipated normal.

TABLE II. Serum Lipid Data in Amaurotic Family Idiocy and Related Disorders.

Diagnosis	Serum total lipids	Serum neutral lipids	Serum phospholipids	Serum free fatty acids	Total cholesterol	Free cholesterol	Cholesterol esters	Ratio, %	Serum lipase, units
	mg %								
Tay-Sachs disease, 4 p, 15 d*	675.0 ± 137.1†	221.0 ± 58.4	155.5 ± 37.0	332.0 ± 56.6	192.5 ± 41.75	41.8 ± 12.04	151.0 ± 32.18	78.4 ± 3.62	1.08 ± .512
	<.01‡	<.01	<.01	.48	.018	<.01	.22	<.01	.28
Gargoylism, 1 p, 5 d	702.0§	175.0	182.0	382.0	271.0	55.0	215.0	79.4	1.15
Schilder's disease, 1 p, 3 d	954.0§	302.0	226.0	411.0	287.0	79.0	208.0	72.6	1.18
Normal subjects	861.0 ± 75.0	365.0 ± 59.0	218.0 ± 15.0	320.0 ± 35.0	220.0 ± 20.0	53.0 ± 7.5	162.0 ± 14.0	75.0 ± 2.5	1.23 ± .06

* p = patients; d = determinations. † Mean ± S.D. ‡ P. § Means.

In our series of 7 cases with AFI, a statistical analysis of chemical serum total protein and electrophoretically determined serum albumin showed significant decreases below normal levels. (Table I). In the 2 control cases, with remotely related degenerative diseases of CNS (Schilder's disease and gargoylism), a relatively normal total protein was noted, although serum albumin values were somewhat decreased. Alpha-2 serum globulin fractions were increased in AFI, in contrast to a marked decrease in serum gamma globulin component. Alpha-1 and beta globulin fractions did not vary significantly from normal values. A rather surprising finding was a normal A/G ratio in AFI, since it is frequently depressed in many degenerative diseases, irrespective of etiology. This is exemplified by lowering of this ratio in the case of Schilder's disease (Table I).

The biochemical studies on the CSF samples done simultaneously with the sera, and employing chemical methods, showed a relatively normal total protein but a lowered alpha globulin fraction and markedly decreased gamma globulin values, as well as diminished gamma globulin/total protein ratios. This decrease in CSF gamma globulin levels has not been observed in a wide variety of other progressive, and generally fatal, neurologic diseases and hence cannot be equated with debilitation alone. In most other CNS diseases (including Schilder's disease, gargoylism, multiple sclerosis, cerebral palsy, hemiplegia, etc.) this CSF protein component is either normal or elevated(20,21).

Because of the intimate association of neuraminic acid with AFI, studies of serum level of this complex amino acid were inaugurated. This substance has been identified as one of the abnormal components of the "ganglioside" which constitutes the anomalous dystrophic accumulation within the involved neurons in AFI(1-3). A definite elevation of neuraminic acid in our 4 cases of AFI was observed. This finding is in keeping with the elevation of this substance previously demonstrated in cerebral parenchyma in this disease(1,22,23). Since the 2 control cases also show an elevation in this component, the specificity of this change in AFI as compared to other neurologic disorders is presently under investigation.

Previous biochemical studies on lipoid dystrophies have dealt mainly with variations in the lipid fractions of visceral organs(24). In a few of these diseases such lipid abnormality is also reflected in alterations of such fractions within the serum. No comparable changes of serum lipids have been reported in AFI to our knowledge. For this reason, biochemical studies of serum lipid partition within total lipids of this disease were performed (Table II). When such results were statistically compared to those derived from normal subjects of comparable age, some marked variations were noted. The total serum lipids, neutral lipids and phospholipids all showed a significant decrease. In the one case of gargoylism, directional changes of a similar nature were recorded. In the instance of Schilder's disease, however, normal or elevated values of these lipid fractions were noted. In studies of the cholesterol fractions, a slight decrease of free cholesterol and slight elevation in the cholesterol ester/total cholesterol ratio were seen. No significant changes were apparent in total cholesterol or cholesterol esters however. Serum lipase and free fatty acid levels were normal. Further studies concerned with lipid and protein metabolism in AFI are now in progress.

Summary. Serial biochemical serum and cerebrospinal fluid (CSF) studies in 7 cases of Amaurotic Family Idiocy (AFI) disclosed some *statistically* significant variation from normal values. Two additional control cases of dissimilar degenerative neurologic illnesses of childhood were also included as control cases. In AFI an elevation in serum electrophoretic alpha-2 globulin fraction was found. Decreased values for serum total protein and albumin, together with markedly depressed gamma globulin results were observed. Serum alpha-1 and beta globulins and A/G ratio findings were normal. Chemical studies of CSF proteins showed decreased alpha and gamma globulins and gamma globulin/total protein ratio values. Serum neuraminic acid was consistently elevated in AFI, as well as in the 2 control cases, when compared to normal values established in this laboratory. Decreases in serum total, neutral and phospholipids were recorded in AFI. A decrease was also observed in serum free cholesterol values. The results in AFI differed from the 2 control cases of degenerative central nervous system disease most markedly with respect to lowered alpha and gamma globulin content of the CSF and with regard to decreased serum total lipids and phospholipids.

We wish to acknowledge the helpful cooperation of Dr. Abram Kanof and Dr. Nathan Epstein for their aid in diagnosis and selection of patients for this study.

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Chemotherapy of Leukemia VII. Effect of Substituted Triazenes on Transplanted Mouse Leukemia.* (22273)

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During routine screening of compounds for possible antitumor effects, a member of a new series of compounds not previously known to possess chemotherapeutic activity, 3,3-dimethyl 1-phenyl triazene (Triazene I), was found by Clarke *et al.*(1) to inhibit growth of Sarcoma 180 in mice. Following this Dagg, Karnofsky *et al.*(2) demonstrated teratogenic effects on the chick embryo and inhibition of growth of Sarcoma 180 and certain human tumors implanted on the chorioallantoic membrane. An evaluation of chemotherapeutic effects of this and related compounds against various strains of transplanted leukemia in mice has been undertaken and the results are herewith reported.

Method. The general technic used for evaluating chemotherapeutic activity of a given drug by means of its ability to prolong survival time of mice with transplanted leukemia has been described previously(3). The 60th to 70th transplant generations of leukemia 82(4), which originated as a spontaneous leukemia in a C58 mouse in October 1953, were used for most of these studies. This transplantable leukemia usually kills the mice in 10 to 15 days after inoculation, with an elevation of total leukocyte count to the 50000 to 200000 level, enlargement of liver and spleen, and some lymphadenopathy. A few studies were conducted on the 10th to 14th generation of leukemia 5471, which arose as a spontaneous leukemia in a C58 mouse in July, 1954. Although morphologically these 2 leukemias are very similar, 82 has always been relatively refractory to folic acid antagonists, whereas 5471 is quite sensitive. In these experiments mice of the Fl hybrid generation of C58 male X Bagg albino female cross were each injected intraperitoneally with 0.1 cc of saline suspension of minced spleen containing one million cells per inoculum. The A-methopterin sensitive (L1210S) and A-methopterin dependent (L1210AD) variants of Leukemia L1210 obtained from Law(5,6) were also used. These leukemias were transplanted by subcutaneous injections into dba mice of minced tumor tissue suspended in isotonic saline. Treatment was initiated 24 hours after inoculation and repeated 3 times weekly for 10 doses over a 23-day period. The mice were observed for development of leukemia and autopsied at death. Triazenes that have been adequately tested and their maximum tolerated doses are listed below. Because of low solubility in water, these compounds were either dissolved in peanut oil (PO) or suspended in 0.5% sodium carboxymethylcellulose (CMC) in isotonic saline as indicated:

I.	3,3-dimethyl-1-phenyl triazene	12-25 mg/kg (PO
II.	3,3-diethyl-1-phenyl triazene	150 mg/kg (PO)
III.	3,3-dimethyl-1-p-nitrophenyl triazene	250 mg/kg (CMC)
IV.	3,3-dimethyl-1-p-tolyl triazene	400-625 mg/kg (CMC)

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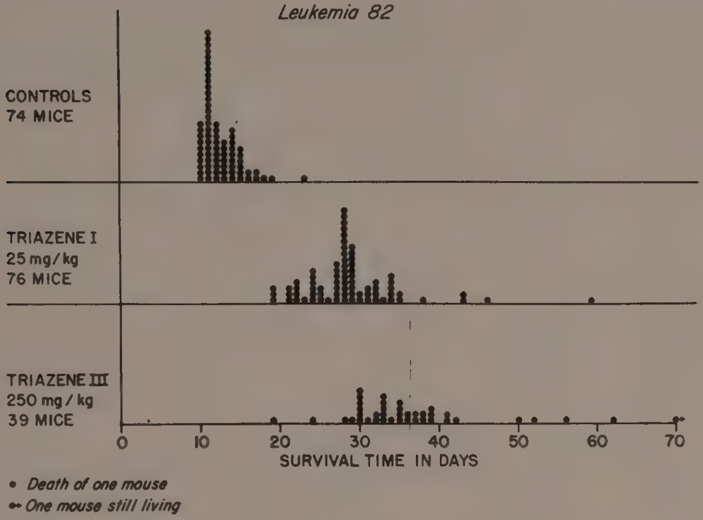

FIG. 1.

Results. The scatter diagram in Fig. 1 shows the relative effectiveness of triazenes I and III in leukemia 82. The mean survival time of the 74 control animals was 12.5 days whereas mice treated with Triazene I at a dose of 25 mg/kg of body weight showed mean survival time of 28.9 days and those receiving Triazene III at 250 mg/kg 35.8 days.

The comparative effectiveness of the various triazenes and various standard agents against leukemias 82, 5471, L1210S and L1210AD is demonstrated in Table I.

It can be seen that Triazene III was somewhat more active against leukemia 82 than Triazenes I and IV and that the diethyl triazene (II), was not as effective.

The triazenes were without therapeutic activity against L1210S and L1210AD. In other experiments using variants of leukemia 82 made resistant to amicetin(7), azaserine (8) or aminonucleoside(7), Triazene I was still effective in prolonging survival time.

Using a technique previously reported(9), massive single doses of Triazene I of 1000, 2000, 4000, and 8000 mg/kg of body weight (5, 10, 20 and 40 times the acute LD_{50} dose respectively) were injected intraperitoneally into mice with far advanced 82 leukemia. Bioassay of spleens from these animals into young mice of the same strain 1-2 hours after injection produced leukemia at all levels. This result is in contrast to the sterilizing effects in the same experiment of single injections of 2 to 10 times the LD_{50} dose of one of the more potent nitrogen mustards 1, 2, 3, 4, tetrakis (bis [2-chloroethyl] amino) butane tetrahydrochloride(9,10) and in contrast to the previously reported sterilizing effect of triethylene melamine (TEM) and other alkylating agents on various strains of mouse leukemia.

Discussion. It has been postulated that the chemotherapeutic activity of triazenes is due to their action as alkylating agents. In this respect it should be noted that they are active on strains of transplantable mouse leukemia (82 and 5471) which are also inhibited by the nitrogen mustard methyl-bis (β-chloroethyl) amine (HN2) but that although Triazene I prevents the transplantability of Sarcoma 180 growing on the chorioallantoic membrane of the chick embryo(2) no sterilizing effect could be demonstrated in mouse leukemia in contrast to the activity of HN2, TEM, and other alkylating agents in this respect. Thus it is evident that certain differences as well as similarities exist between the triazenes and the typical alkylating

TABLE I. Comparative Effects of Triazenes on Various Strains of Mouse Leukemia. Mean survival time in days and range.

	Dose, mg/kg	Leukemia 82				Leukemia 5471	Leukemia L1210S	Leukemia L1210AD
		Exp. 1	Exp. 2	Exp. 3	Exp. 4			
Controls	—	11.2 (11–12)	14.9 (12–17)	14.3 (12–19)	11.8 (10–14)	15.2 (14–17)	13.3 (12–14)	15.4 (8–20)
Triazene I	25	27.8 (22–38)	31.4† (33–50)	30.4 (24–43)	24.1 (19–29)	32.3 (24–49)	13.9 (12–15)	16.4 (13–20)
	12.5	34.2* (22–50)	37.2* (30–50)	42.6‡ (29–50)	39.7 (33–45)	—	—	15.7 (9–20)
II	150	15.5 (12–18)	—	—	—	—	—	—
III	250	34.4* (19–50)	—	—	43.2 (33–62)	—	14.2 (14–15)	14.8 (9–20)
IV	62.5	21.5 (15–28)	—	—	—	—	—	—
	40	—	35.6 (27–44)	—	—	—	—	—
HN2	1	19.0 (12–22)	—	24.6 (22–28)	—	—	—	13.9 (11–17)
Urethane	1000	—	—	22.3 (20–27)	27.3 (20–34)	22.7 (19–28)	—	—
A-Methopterin	3	—	—	—	—	45.7 (35–84)	26.1 (21–37)	13.5 (11–16)

* 1 living at 50 days. † 2 living at 50 days. ‡ 3 living at 50 days.

agents.

In comparative studies on triazene derivatives, addition of the nitro group in the para position of the phenyl group (III) decreased markedly the toxicity of the compound for the host mouse and increased somewhat its relative chemotherapeutic effectiveness against leukemias 82. It was not an effective agent however, against the Amethopterin sensitive or Amethopterin resistant variants of leukemia L1210 which were also refractory to the parent compound (I). Lack of effect of this derivative (III) on development of the chick embryo was attributed by Dagg *et al.*(11) to the fact that in the 4-day-old chick embryo in contrast to the mouse, the addition of the nitro group in the para position of the phenyl group increased rather than decreased the overall toxicity of the compound and thus perhaps made it impossible to give large enough doses to demonstrate a teratogenic effect.

The fact that these triazenes were active against Leukemia 82 which is initially refractory to treatment with Amethopterin and also against lines of Leukemia 82 made resistant to Azaserine, Amicetin, or Aminonucleoside indicates that these compounds are acting through a different mechanism in producing their chemotherapeutic effects.

The above mentioned studies of Clarke *et al.*(1) of Dagg, Karnofsky, *et al.*(2) and the presently reported studies on mouse leukemia suggest that this new series of compounds be given clinical evaluation against neoplastic disease. Such studies are now in progress.

Summary. 1-phenyl 3, 3-dimethyl triazene has been demonstrated to prolong survival time of mice with transplanted Leukemias 82 and 5471 but not with an Amethopterin sensitive or an Amethopterin resistant variant of Leukemia L1210. A closely allied derivative, 1 - p - nitrophenyl - 3,3-dimethyl triazene has shown relatively greater chemotherapeutic activity against Leukemia 82 but was inactive against the two variants of Leukemia L1210. These compounds were also active against strains of Leukemia 82 which were either initially refractory to or made resistant to Amethopterin, Azaserine, Amicetin, and Aminonucleoside. Further therapeutic studies on this class of compounds are indicated.

We would like to express our appreciation to Dr. Christian S. Rondestvedt of the University of Michigan and to Dr. Ralph Barclay of the Sloan-Kettering Institute for supplying several compounds used.

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Effect of Adrenalin on Resistance of Adrenalectomized Rats to Certain Toxic Agents. (22274)

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Prior to 1917, the secretion of the adrenal medulla was believed to be the entity which rendered the adrenal glands indispensable for maintenance of life(1). Following the demonstration that epinephrine-free cortical extracts maintained normal blood sugar levels in adrenalectomized rats(2), the role of the medullary hormone has generally been regarded as of minor importance, if any. In searching for a rapid method of assay for DOC-like activity of steroids, it was observed that adrenalin rather than cortical steroids conferred resistance to administration of certain toxic agents in adrenalectomized rats. The sequence of events which led to this finding is here described. On the premise that adrenalectomized animals exhibit decreased tolerance to administered potassium(3), the proposed method of assay was to determine the amounts of cortical steroids which would provide increased resistance to the injection of potassium chloride.

Methods. Intact and adrenalectomized male albino Sprague-Dawley rats, one-half of each group having been treated with desoxycorticosterone acetate, 1 mg rat/day up to 7 days, were injected subcutaneously with 0.5 cc of 10% potassium chloride solution every 10 minutes until death, and the number of injections given up to the time of death was recorded. It was noted that adrenalectomized animals succumbed after only one-half as many injections as those given intact animals, and that even intensive pretreatment with DCA did not diminish the susceptibility of adrenalectomized rats to injected potassium chloride.

Results. Since, in some of the adrenalectomized rats, convulsions suggestive of hypoglycemic shock were noted after only one or 2 injections of KCl, glucose determinations were made on blood collected from the heart at the instant of respiratory failure. These values are shown in Table I.

TABLE I. Blood Sugar of Intact and Adrenalectomized Rats Injected with KCl.

Blood sugar (mg %)*	Untreated	KCl-treated
Intact	135	275
Adrenalectomized	85	45

* Pool of 4 animals.

The blood sugar values confirmed the impression that adrenalectomized rats were indeed in a hypoglycemic state, and also showed that intact animals exhibited an increase in

TABLE II. Effects of Adrenalectomy, Adrenal Demedullation, and Administration of DCA, Cortisone, and Adrenalin on Sensitivity to KCl.

No. of inj.	4	5	6	7	8	9	10	11	12	13	14	15	16	Blood sugar (mg %) Initial	Blood sugar (mg %) Terminal
No. of deaths:															
Intact			2	3	4		1	2						130	270
Adx.	5	3	1	2	1									115	40
" + DCA	4	3		3	2									155	80
" + cortisone	1	1	3	3	2	1	1							135	75
Demedullated	1	2	2	3	3			1						—	125
Adx. + adrenalin				2	4	2			1	2			1	110	240

blood sugar. It was thereupon decided to investigate whether the hypoglycemic response of the adrenalectomized rats could be modified by the administration of glucocorticoids, and if so, whether their sensitivity to KCl could thereby be reduced. In addition, since the ablation of adrenals deprives the animal of another hyperglycemic factor, the medullary secretion, the study was extended to include the effect of adrenalin administration. One group of adrenalectomized rats was treated for 7 days with 1 mg cortisone acetate injected subcutaneously/rat/day. On the 8th day they were injected with KCl as in the foregoing test. Another group of adrenalectomized rats was injected with a solution of KCl, each 0.5 cc containing 10 γ of adrenalin chloride. A third group consisted of rats whose adrenals had been enucleated 1 month previous to the test. The remaining groups included DCA-treated adrenalectomized rats, and untreated intact and adrenalectomized controls. Blood sugars were collected terminally.

The results (Table II) confirmed the previous findings that untreated adrenalectomized rats tolerated less KCl than intact animals and that DCA-treated adrenalectomized rats showed the same sensitivity to KCl as untreated adrenalectomized animals. They also demonstrated that cortisone-treated adrenalectomized rats and demedullated rats were only slightly less sensitive to KCl than adrenalectomized animals and that adrenalin-treated adrenalectomized rats tolerated KCl as well as or possibly better than did intact rats. Hyperglycemia was present in the intact and in the adrenalin-treated rats; the cortisone- and DCA-treated groups and the demedullated rats showed a lower blood sugar level than the intact rats. The untreated adrenalectomized animals exhibited hypoglycemia.

In view of the protection given by adrenalin, this treatment was tested against 2 other arbitrarily selected toxic agents, formaldehyde and sodium arsenite. The former was injected subcutaneously in doses of 0.1 cc of a 10% solution; the latter in 0.5 cc doses of a 1% solution. The results (Tables III and IV) show that adrenalin increased the resistance of adrenalectomized rats to both reagents. In the case of formaldehyde, however, the adrenalin-treated adrenalectomized rats were still more sensitive than the intact animals, while with sodium arsenite, the adrenalin-treated adrenalectomized rats were, if anything, somewhat more resistant than the intact rats.

TABLE III. Effect of Adrenalin in Formaldehyde Poisoning.

No. of inj.	5	6	7	8	9	10
No. of deaths:						
Intact			1			*
Adrenalectomized	3		1		1	
Adx. + adrenalin		1	1	1	1	1

* Of 5 intact animals, all but one were still alive after 10 injections, although they were in a state of collapse.

TABLE IV. Effect of Adrenalin in Sodium Arsenite Poisoning.

No. of inj.	3	4	5	6	7
No. of deaths:					
Intact	1	6			1
Adrenalectomized	4	3			1
Adx. + adrenalin			6	2	

Although the increased resistance in adrenalin-treated animals was always accompanied by a rise in blood sugar, the hyperglycemic

TABLE V. Comparative Effects of Adrenalin and L-arterenol.

No. of inj.	3	4	5	6	7	8	9	10	11	12	Blood sugar (terminal) mg %
No. of deaths:											
Adx. + KCl	1	6	3	2	1						105
" + adrenalin		1	2	2	2	1	1	2	1	1	185
" + L-arterenol			4	3	3		2	1			135

state appeared to be merely a measure of adrenalin activity and not the mechanism responsible for survival. The blood sugar level in the adrenalectomized rats, even terminally, was never so low as to permit an assumption that hypoglycemia was the immediate cause of death. It seemed likely that the primary effect of adrenalin was to resist circulatory collapse. Since l-arterenol (nor-epinephrine) is known to maintain vasomotion while only slightly raising the blood sugar level, the effects produced by this agent should reveal the significance of the hyperglycemia, if any. L-arterenol was therefore administered in the same manner as was adrenalin. The results (Table V) showed that adrenalin and l-arterenol had comparable effects.

It appeared, then, that the supportive effect of the medullary hormone was primarily in maintaining vascular tone, and that the blood sugar values were a rough index of the level of circulating adrenalin.

Finally, a study was made of the effects of blocking the activity of adrenalin. Two sympatholytic agents were tested: Gynergen (ergotamine tartrate) administered in doses of 0.1 cc each containing 5 γ with each injection of adrenalin; and dibenamine in doses of 0.1 cc each containing 1 mg.

It is seen from Table VI that the dose level of Gynergen used was not adequate to block the action of adrenalin or of the intact adrenal medulla. It did, however, suppress hyperglycemia to some extent. Dibenamine, on the other hand, did not affect the usual blood sugar response to adrenalin, but abolished the resistance provided by the latter.

Discussion. The findings indicate that the administration of adrenalin provides substantial protection to adrenalectomized rats against some toxic agents, and that, in the case of KCl and sodium arsenite, the prolongation of life may actually surpass that of intact rats. The production of hyperglycemia is evidently not the mechanism by which the medullary hormone participates in the resistance against these agents; animals treated with adrenalin-antagonists show enhanced susceptibility to intoxication in spite of hyperglycemia.

The relative importance of the adrenal cortex and the medulla in the maintenance of vascular tone is not established. Zweifach, *et al.*(4) obtained in adrenalectomized rats

TABLE VI. Effects of Gynergen and Dibenamine.

No. of inj.	5	6	7	8	9	10	11	12	13	14	15	16	17	Blood sugar, mg %
No. of deaths:						Adrenalectomized rats								
KCl	3		2											120
" + A*							2	1		1	1			200
" + A + G									2	2		1		170
" + A + D			2	1	1	1								230
						Intact rats								
KCl				1		1	2	1						260
" + A										1	1	1	2	360
" + G							3	1	1					190
" + D			1	1	2	1								250

* A = Adrenalin; G = Gynergen; D = Dibenamine.

restoration of the normal state in the vascular bed with cortical steroids. Works of Swingle and others(5,6) point to the role of the cortex in traumatic shock, while Huizenga, *et al.* (7) report on the ineffectiveness of cortical hormones in hemorrhagic shock. Remington (8) found that intact dogs given a large dose of dibenamine succumbed to minor hemorrhages, and that the cardiovascular changes in these animals resembled those of animals in terminal adrenal insufficiency or acute crises following bilateral adrenalectomy. Ramey, *et al.*(9) found that ACE potentiates the blood pressure response to norepinephrine in adrenalectomized dogs. We, however, were unable to confirm this in rats. Fritz and Levine(10) believe that cortical steroids are necessary for the efficient response of blood vessels to sympathin E.

Whatever may be the mechanism, the results obtained by us, together with the observations that adrenalin injection increases the resistance of adrenalectomized animals to histamine(11-13) suggest that at least under the conditions of the present experiments, the presence of the adrenal medulla is of primary importance.

Summary. 1. Administration of adrenalin prolongs survival time of adrenalectomized rats injected with KCl, formaldehyde, and sodium arsenite. 2. Under conditions of our experiments, adrenalin was more effective than cortisone in providing protection against these agents. 3. This protective effect is abolished by simultaneous administration of sympatholytic drugs. 4. The mechanism of protection appears to be through the prevention of vascular collapse. 5. It is suggested that the medullary secretion is an important factor in resisting traumatic shock.

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Studies on Platelets. XVI. Glutamic Oxaloacetic Transaminase Activity of Human Platelets.* (22275)

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Glutamic oxaloacetic transaminase (GO-T) is a specific enzyme concerned with the synthesis of glutamic and oxaloacetic acids through the transfer of α-amino nitrogen of aspartic acid to α-ketoglutaric acid. Its presence has been demonstrated in serum of many animals and man, as well as in many animal tissues(1-4). It was observed by Karmen *et al.*(4) that human blood hemolysates

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contained GO-T activity greatly in excess of that of human serum. This observation suggested the presence of GO-T in formed elements of the blood. Due to our interest in platelets, a study was done to determine the GO-T activity of these bodies.

Methods. 1) *Determination of GO-T activity* was carried out as described by Karmen *et al.*(4). A minor modification was introduced consisting in the use of 0.03 ml of malic acid reagent instead of 0.1 ml as indicated in the original technic. This modification has given results quite comparable to those obtained with the original method(5). Experiments were run in triplicate. The reacting mixture was incubated for 10′ and readings were taken at 5′-10′-15′ after addition of the aspartic acid reagent. Values given in this study are, therefore, the average of nine figures obtained for each sample. 2) *Preparation of platelet-rich and platelet-poor plasma; and of platelet suspensions in saline* was carried out as described(6). Technics differed, however, in small details. Standard ACD solution (1/5 volume) was used as anticoagulant instead of EDTA-Na_2. Blood was freshly collected from healthy donors in plastic containers and processed without delay. Volume of saline used in washing platelets was identical to that of the platelet-rich plasma originally used. Platelet suspensions were adjusted to various concentrations by addition of suitable volume of saline solution. Platelets were counted directly(7) with the use of phase microscope. All preparations were assayed for enzymatic activity within 8 hours of blood collection. 3) *Preparation of water soluble platelet extract.* This original technic is being described(8). To packed, fresh, twice washed platelets from 50 ml of fresh human blood, 6 ml of distilled water and 5 ml of precooled ethyl ether were added. After centrifugation at 3,500 rpm/30′ at 4°C, three well differentiated layers were obtained: an upper layer, containing ethyl ether and lipoid substances; a middle one containing a precipitate, probably composed of protein material and stroma, and a bottom one containing a water-soluble protein. After separation of the supernant ether layer, the middle and lower layers were brought back with distilled water to the volume of platelet-rich plasma originally used. Centrifugation at 3,200 rpm/30′ at 4°C brought to sharp separation of a top layer containing insoluble protein material and stroma from a water-clear bottom layer. The bottom layer, representing approximately 19/20 of the total volume was transferred by suction to another container, leaving behind the top layer. Enough water was then added to full reconstitution of volume.

TABLE I. Glutamic Oxaloacetic Transaminase Activity in Various Preparations from Human Platelets.*

Preparation	No. of platelets/mm³ × 10⁻³	Units of GO-T activity
Normal plasma	235 ± 25	37.5 ± 7.5
Platelet-poor plasma	10 (approx.)	20.8 ± 2.0
Platelet suspension (saline)	2000 ± 200	255.0 ± 10.5
	470 ± 15	61.5 ± 3.5
	120 ± 24	43.0 ± 2.0
	40 ± 5	28.5 ± 1.5
Washings of sedimented platelets		10.5 ± 1.6
Water soluble fraction (see text)		104.2 ± 10.0

* Values calculated on basis of 5 different experiments.

Results. (Table I). (a) Plasma containing an adequate number of platelets showed greater GO-T activity than platelet-poor plasma; (b) Slight GO-T activity was recovered from washing of platelets after their separation from plasma by high speed centrifugation. This may have represented GO-T activity of plasma which had remained absorbed into the platelets; (c) The transaminase activity of isolated platelet preparations containing discrete, separated bodies was in gross proportion to their number; (d) The water soluble protein obtained from platelets (bottom layer) appeared to possess high GO-T activity.

Discussion. There seems to be no question that platelets represent a source of GO-T in human blood. In view of the present uncertainty as to whether a factor discovered in platelets is an intimate constituent of these bodies or is more simply carried by them, it became necessary to study the "location" of GO-T in human platelets. Low GO-T activity was found in washings from separated platelets. On the other hand, high activity was found in a water soluble preparation from platelets after treatment with ether. Ether appeared to destroy the physical integrity of platelets while removing most of their lipoid constituents. Both findings, therefore, suggested that GO-T was an intimate constituent of platelets, presumably a part of the protein, water-soluble fraction.

It appears premature to discuss the significance of the finding of GO-T in platelets as well as the possible relationship of the enzyme to the role of platelets in the hemostatic mechanism.

Summary. 1) Platelets are a source of glutamic oxaloacetic transaminase in human blood. 2) The enzyme is an intimate constituent of platelets, being found principally in a water soluble fraction of platelets after separation of lipoids by ethyl ether.

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Amperometric Titration of -SH Groups in Purified Clotting Factors By Improved Method. (22276)

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In studies on specific reactive groups of proteins involved in the coagulation of blood, it was observed that compounds commonly used to alter –S–S– bonds caused inhibition of clotting activity of purified bovine prothrombin, whereas those used to alter –SH groups did not(1). No –SH groups were detected in purified prothrombin, thrombin or fibrinogen, nor during the conversion of prothrombin to thrombin. In these experiments, quantitative –SH measurements were made by the argentimetric amperometric method using an ethanol-ammoniacal titration mixture(1). Benesch, Lardy and Benesch(2) recently have emphasized that this method, as applied to protein –SH groups, is open to criticism on the grounds that the high pH of the NH_4OH-NH_4NO_3 buffer used may denature proteins and accelerate the oxidation rate of –SH groups, that the ammonium ions may effect total disappearance of some –SH groups, and that the alcohol, usually necessary to obtain sharp end points, also denatures proteins. It therefore was possible, although unlikely, that the absence of detectable –SH groups in the purified clotting products could be attributed to these factors.

In this same paper, Benesch *et al.* introduced a modification of the amperometric method which overcame these objections. Instead of ammonia as the agent to form the silver complex, they employed tris-(hydroxymethylaminomethane) which made it possible to titrate protein –SH groups in aqueous, buffered solutions at pH 7.4. Such solutions had the significant additional advantage of exerting no demonstrable drastic effects on proteins, thereby allowing the study of –SH groups of proteins in the native state and a more precise determination of the relative accessibility of these groups after treatment with denaturing agents. They further demonstrated that the Tris method is accurate, sensitive and highly specific for protein –SH groups.

Methods and materials. Several highly active samples of each of the purified prothrombin, citrate thrombin, biothrombin, autoprothrombin I and fibrinogen, all of bovine origin,* and of bovine fibrinogen (Armour and Co.) and Thrombin Topical (Parke, Davis and Co.) were titrated in triplicate for –SH groups using the Tris argentimetric amperometric method(2). The proteins were dissolved in redistilled, deionized water, as were all reagents, and they were made up to a concentration of from 5 to 10 mg/ml. Reduced glutathione was used as a standard control. Each of the proteins was titrated in its native state, and following denaturation with half saturated urea, half saturated guanidine hydrochloride and by boiling for 30 minutes, respectively.

Results. Under none of the above conditions were any –SH groups detected in the purified clotting factors, thus substantiating the results obtained indirectly with the use of –SH blocking agents and directly with the ammoniacal-ethanol amperometric method (1).

Discussion. Ethanol-ammoniacal argentimetric amperometric titrations for quantitative measurement of –S–S– bonds had been carried out on each of the above listed proteins(1,3) with results which indicated that all but fibrinogen contained 43 μM –S–S–/100 mg N; prothrombin contained 4 moles –S–S–/mole. Since the principle of the method(4) is the sulfitolysis of protein –S–S– with the release of 1 mole –SH for each mole –S–S–, the objections to the titration of –SH groups in an ethanol-ammoniacal medium were applicable to this method. Repeated attempts to adapt the Tris method to the measurement of protein –S–S– resulted in failure, even though accurate, reproducible values were achieved in the measurement of –S–S– bonds of oxidized glutathione. Although the indicated objections are valid, the extensive control studies with oxidized glutathione and with insulin, and the good agreement with the amino acid analysis of purified bovine prothrombin and of fibrinogen(1,4) would indicate that the original –S–S– values are correct, and that the "ammonium effect" is not operative with the protein products listed above.

Summary. Since the ethanol-ammoniacal amperometric method for quantitative measurement of –SH groups in proteins is susceptible to valid criticism, measurements were repeated on certain clotting proteins using the Tris buffer system. This procedure also resulted in the inability to detect –SH groups in these proteins.

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Acetal Phosphatides in the Adipose Tissue of Newborn Rats. (22277)

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Since the discovery of acetal phosphatides by Feulgen and Voit(1) a considerable amount of work has been done upon the distribution and isolation of acetal phosphatides in tissues. Few investigators have made quantitative chemical studies of the metabolic role of these compounds. Stetten and Schoenheimer(2), basing their conclusions upon experiments utilizing deuterium labeled compounds, have indicated that fatty aldehydes may be intermediates in the interconversion of fatty acids and fatty alcohols. On the other hand, Ehrlich and Waelsch(3), also using deuterium labeled fatty acids, concluded that fatty aldehydes were not directly involved in the overall synthesis or transport of fatty acids. The suggestion that acetal phosphatides are involved in both the anabolism and catabolism of fatty acids has been made by Danielli(4), Fawcett(5), Schäfer (6) and Möckel(7). Möckel, basing his conclusions upon a series of histological studies of the adipose tissues of newborn rats and of rats subjected to starvation, proposed that the anabolism and catabolism of neutral fat follows the pathway of fatty aldehyde to acetal phosphatide.

Because of the need for quantitative chemical data on the changes in acetal phosphatide content of tissues actively engaged in lipid metabolism it was decided to investigate the changes in acetal phosphatide in relation to phospholipid and total lipids in the adipose tissues and livers of newborn rats over the first 21 days of life.

Materials and methods. Newborn rats at 1, 2, 3, 5, 8, 15 and 21 days of age were sacrificed by decapitation. The livers and samples of subcutaneous adipose tissue from the pectoral and pelvic regions were removed and freed from foreign tissue. In age groups 1, 2, 3, 5 and 8 days, tissues of 2 or 3 animals were pooled. In pooling tissues, care was taken to assure that the number of animals taken from any one litter was the same for each age group. The lipid of the tissues was extracted by the method of Rice and coworkers(8). The extracts were then analyzed for acetal phosphatide, total phospholipid and total lipid. Acetal phosphatides were determined by the method of Feulgen, Boguth and Andresen(9). Phospholipid phosphorus was determined by the procedure of Fiske and Subbarow(10). Total lipids were determined gravimetrically.

Results. The results are summarized in Table I. This table represents the average results from 2 separate series of experiments. In each series the changes noted were parallel and of the same order of magnitude. Preliminary experiments where only acetal phosphatides were determined had shown that

TABLE I. Lipid Changes in Adipose Tissues and Livers of Rat during First 3 Weeks of Life.*

	Adipose tissue			Liver		
Age, days	Total lipid, %	Phospholipid phosphorus, μmoles/g	Acetal phosphatide, μmoles/g	Total lipid, %	Phospholipid phosphorus, μmoles/g	Acetal phosphatide, μmoles/g
1	19.1	28.10	1.72	7.31	25.2	.401
2	29.0	25.40	1.02	4.10	19.5	.508
3	42.5	17.90	.64	4.67	18.8	.392
5	45.8	4.90	.24	5.43	27.3	.406
8	61.5	1.24	.05	5.41	28.7	.383
15	59.1	3.37	.08	4.63	31.2	.472
21	68.9	1.74	.03	4.98	36.1	.367

* All values in terms of wet wt of tissue.

there was some variation among litters in values obtained from animals at the first 3 age groups. This variation was associated with the number of animals in the litter, since animals from the larger litter showed a higher initial acetal phosphatide content in the adipose tissue. The changes associated with increased age for each litter were parallel and of the same order of magnitude. In the experiment described above and where tissues were pooled, the variation among litters was minimized by keeping the number of animals from any one litter the same for each age group.

Discussion. The changes in the lipid fractions of the liver with increased age in newborn rats were within normal adult limits and little significance can be attributed to them. These data, however, do not exclude the possibility that acetal phosphatides may be involved in the lipid metabolism of the liver by means of a turnover mechanism. In adipose tissue, on the other hand, the maximum changes occurred during the first 5 days of life. The initially high levels of acetal phosphatide and total phospholipid decreased rapidly to only trace amounts as the adipose tissue filled with fat. The results confirm and extend the observation of Möckel(7). The inverse changes in acetal phosphatide and phospholipid, as compared to total lipid, indicate that they are in some way involved in the laying down of fat in adipose tissue. Acetal phosphatides could be involved directly in this mechanism or they could be involved in the synthesis of other phospholipids which could then play some role in the fat metabolism of adipose tissue.

Summary. The changes in the acetal phosphatide, phospholipid and total lipids have been followed in the adipose tissues and livers of newborn rats over the first 3 weeks of life. As the adipose tissue fills with fat, the acetal phosphatide and total phospholipid, initially quite elevated, decrease rapidly to only trace amounts by the fifth to eighth day of life. These results indicate that acetal phosphatides and phospholipids are involved in some manner in the laying down of fat in adipose tissue.

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Effect of Para-Aminosalicylic Acid on Serum Isoniazid Levels in Man.
(22278)

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Previous investigations(1,2) with chemical methods have shown wide variations in the rate of metabolic alteration of isoniazid (INH) from person to person, but a relatively constant rate in any one individual. This observation is of clinical importance because only the free, unaltered isoniazid has significant antimicrobial activity against tubercle bacilli(3). One of the major pathways of metabolic disposition of INH is by acetylation(1). *In vitro* experiments by Johnson (4) have shown that para-aminosalicylic acid (PAS) inhibits the acetylation of INH by a competitive mechanism.

The purpose of this report is to present observations on the application of a specific microbiologic assay technic to determine the effect of PAS on the level of antimicrobially active INH in human subjects.

Materials and methods. The technic of microbiologic assay for INH was as follows: 1 ml of serum from aseptically collected specimen of clotted venous blood was added to 9 ml of oleic acid-albumin medium ST-T* containing 10 μg PABA/ml. From this tube of 1:10 serum dilution, 2.5 ml aliquots of serum dilutions of 1:10, 1:20 and 1:40 were prepared in duplicate in ST-T medium in screw-capped culture tubes. Each of 6 tubes for any one determination was then inoculated with 0.1 ml of fully-grown, dispersed, stock culture of an INH-susceptible, streptomycin-resistant strain of tubercle bacilli (H37RvS-RSM) grown in ST-T medium containing 0.05% Tween 80 and 10 μg streptomycin/ml. The inoculated tubes, along with standard control test of susceptibility of the strain to known concentrations of INH (0, .02, .04, and .08 μg INH/ml), were incubated at 36°. After 5 days, a Ziehl-Neelsen stained smear from each tube was prepared and the tubercle bacilli examined for loss of acid-fastness (AF). This is a specific test for antimicrobially active INH as neither human sera nor any known antimicrobial agent other than INH has this effect on tubercle bacilli(6). The results were read as follows: (1) No loss of AF at 1:10 dilution (<0.3 μg INH/ml); (2) Moderate loss of AF at 1:10 dilution, no loss at 1:20 (0.3 μg INH/ml); (3) Marked loss of AF at 1:10, no loss at 1:20 (0.4-0.6 μg INH/ml); (4) Moderate loss of AF at 1:20, no loss at 1:40 (0.7 μg INH/ml); (5) Marked loss of AF at 1:20, no loss at 1:40 (0.8-1.4 μg INH/ml); (6) Moderate loss of AF at 1:40 (1.5 μg INH/ml); (7) Marked loss of AF at 1:40 (1.6 μg or more INH/ml). Preliminary studies with known amounts of INH added to human sera permit assignment of these values for INH equivalents in μg/ml. It was also shown that addition of as much as 100 μg streptomycin/ml and 1000 μg PAS/ml to undiluted human sera did not interfere with this microbiologic assay. A total of 76 assays were performed on 25 patients under treatment for tuberculosis. Patients were selected from a group who did not maintain serum INH levels in excess of 0.3 μg/ml 6 hours after oral loading dose of 4 mg INH/kg in tablet form (Nydrazid, Squibb). This standard loading dose regimen required that

TABLE I. Effect of PAS on Microbiologic Assay of Serum INH, in 25 Patients.

	Loading dose, mg/kg	Without PAS, μg INH/ml	With PAS, μg INH/ml	Result*
H.W.	4	<.3	.8–1.4	+
T.H.	”	”	.4– .6	”
C.H.	”	”	.8–1.4	”
W.C.	”	”	.4– .6	”
W.D.	”	”	”	”
W.S.	”	”	.3	”
P.M.	”	”	.4– .6	”
G.R.	”	”	<.3	0
M.P.	”	”	”	”
C.M.	8	”	.8–1.4	+
E.R.	”	.4– .6	”	”
M.B.	”	”	”	”
A.B.	4	<.3	.4– .6	”
	”	.3	”	”
S.P.	”	<.3	.8–1.4	”
	”	”	.3	”
M.H.	8	”	.4– .6	”
	”	”	.8–1.4	”
F.H.	”	.4– .6	”	”
	”	”	”	”
B.S.	4	<.3	.7	”
	8	.3	.8–1.4	”
J.E.	4	<.3	<.3	0
	8	”	.4– .6	+
S.C.	4	”	”	”
	8	.4– .6	.8–1.4	”
A.J.	4	<.3	.4– .6	”
	8	.4– .6	.8–1.4	”
B.Y.	4	<.3	.4– .6	”
	8	.3	”	”
R.C.	4	<.3	<.3	0
	8	.4– .6	.8–1.4	+
M.B.	4	<.3	.4– .6	”
	8	.8–1.4	.8–1.4	0
M.S.	4	<.3	<.3	”
	8	.3	.4– .6	+
M.G.	4	<.3	<.3	0
	8	”	”	”

* + = Increase; 0 = No detectable change.

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* Same as "basic medium" previously described(5) except for addition of 0.4 g sodium citrate, 0.01 g ferric ammonium citrate and 2 g glycerol/l; 0.5% oleic acid-albumin complex plus 0.2% glucose (final concentrations) were added aseptically after autoclaving basic medium.

the patient have received total daily dose of INH from 8 to 16 mg/kg for at least one day prior to test and that, except for streptomycin, he receive no antimicrobial agent other than INH. The INH was given at 9 a. m. and blood was collected at 3 p. m. When PAS (Hellwig, PAS Sodium tablets) was administered, it was given in a total daily dose of 10 g of free PAS, 2.5 g at 9 a. m., 12 p. m., 4 p. m. and 8 p. m. Each study was performed under the same conditions with or without PAS which was given or withheld for 5 days prior to each determination.

Results. The results are listed in Table I. Twenty-two of the 25 patients showed a detectable increase in their microbiologically active serum levels of INH when PAS was also given. Repeat determinations with the same loading dose of INH were performed on 4 patients. They showed increases each time that PAS also was administered. Repeat determinations with different loading doses of INH were performed on 9 patients. Five were done because there was no detectable change in the first study. In 4 of these 5 subjects there was a detectable increase in INH level when PAS was added. The absence of a detectable increase in 3 of the 25 patients studied did not necessarily mean that PAS had no effect in these individuals for the minimum serum dilution employed (1:10) did not permit detection of possible changes below 0.3 μg INH/ml.

Discussion. The biologic phenomenon of one drug raising the blood level of another drug is not uncommon. The results of the present study demonstrate that concurrent administration of PAS with INH raises the serum concentration of INH when measured 6 hours after administration of INH. In view of Johnson's observation(4), it appears probable that PAS achieves this effect by decreasing the rate of acetylation of INH to the antimicrobially inactive acetyl derivative.

It seems fair to assume that delivery of effective concentrations of INH to multiplying populations of tubercle bacilli in tuberculous patients is determined in part by the concentration of INH in the circulating blood. Therefore, the problem of adequate dosage of INH presents itself in those tuberculous patients who dispose of INH so rapidly that effective antimicrobial levels cannot be achieved or maintained. The present studies show that these levels can be elevated by increasing the dosage of INH or by adding PAS.

The therapeutic superiority of treatment with INH plus PAS over INH alone in human beings(7) may be attributable not only to the antimicrobial activity of PAS itself, but also to the delivery of higher concentrations of INH to multiplying tubercle bacilli.

Summary. A method for the microbiologic assay of INH in the presence of high concentrations of streptomycin or PAS is described. The concurrent oral administration of PAS with INH resulted in detectable elevation of the antimicrobially active INH level in the blood serum of 22 of 25 tuberculous patients. The significance of this effect is discussed.

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Survival of *Histoplasma capsulatum* in Experimental Histoplasmosis in Mice.* (22279)

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Previous studies(1,2) have demonstrated that fatal infections following intraperitoneal inoculation of white mice with yeast phase *H. capsulatum* occur within a 30-day period. After this "acute" period very few of the survivors died within the ensuing year. This study was conducted to determine the fate of *H. capsulatum* after inoculation into white mice.

Materials and methods. Three groups of white Swiss male mice were used in these studies. The intraperitoneal route of inoculation was used throughout. At 8 weeks of age one group received 7 million heat-killed cells, while a second group was inoculated with 0.7 million live yeast-phase organisms in 0.5 ml of mucin, as previously described(2). Following a 6-week period, the "immunized" group and survivors of the second group were challenged with 35 million organisms as was a third control group of 14-week-old mice. At various intervals up to 53 weeks after infection, 8 to 14 mice were sacrificed and cultures obtained. From each animal the whole spleen, heart, right kidney and right lung, and a 1 cm portion of liver were removed aseptically. Each organ was ground in a sterile mortar containing 2 ml of sterile physiologic saline, after which 1 ml of the ground tissue was inoculated on each of 2 Sabouraud's agar plates containing 80 units and 0.5 mg per ml each of penicillin and streptomycin, respectively. The plates were sealed with parafilm, incubated at room temperature, and read at 15 and 30 days. Those cultures showing a solid growth of *H. capsulatum* were arbitrarily designated 4+, heavy but not solid growth as 3+, more than 40 colonies as 2+ and less than 40 as 1+. The sum of the concentrations divided by the number of plates counted was designated as the ARC (average relative concentration of organisms).

Results. Table I shows the results obtained in mice receiving a single challenge dose of 35 million organisms (Group I). During the first 5 weeks all of 14 mice showed positive cultures from the lungs, liver and spleen, while the heart's blood and kidney yielded 11 and 10 positive cultures, respectively. A relatively higher concentration of organisms was observed in the spleen (2.7), liver (2.4) and kidney (2.0) than in the lung (1.7) and heart's blood (1.5). Of 12 mice sacrificed between 6 to 10 weeks after inoculation, positive cultures were obtained from the spleen and liver in 11 and the kidney in 10, while the heart and lungs yielded only 5 and 2 positive cultures, respectively; the average concentration of organisms was likewise higher in the reticuloendothelial tissues (2.6 to 3.2) as compared to that observed in the heart's blood (1.3) and lung (1.0). A similar relationship was observed in 16 mice between 11 to 15 weeks except for only 5 positive cultures from the kidney as compared to 12 and 14 from the spleen and liver, respectively. After 16 weeks no organisms were recovered from the lungs of 38 mice studied while only 1 showed a positive heart's blood culture. This was in contrast to the kidneys, liver and spleen which yielded 42.8, 21.4 and 35.7% positive cultures, respectively, between 16 to 30 weeks, and 35.7, 21.4 and 14.3%, respectively, between 31 to 45 weeks. During the 46- to 53-week period positive cultures were obtained only from the spleen and kidney of 1 of the 10 mice necropsied.

The results in mice previously "immunized" with 7 million heat-killed cells (Group II) were essentially similar as can be seen in Table I. Again it was noted that after 16 weeks the lungs and heart's blood had been cleared of the organisms. The only suggested difference was the relative decrease of the average concentration of organisms in the

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TABLE I. Survival of *H. capsulatum* in Mice following Primary Challenge, Reinfection, and after "Immunization."

		Positive cultures								
		Group I*			Group II*			Group III*		
Tissue	Wk post-inoc.	No.†	%	ARC‡	No.	%	ARC	No.	%	ARC
Heart	1– 5	11/14	78.6	1.5	6/8	75.	1.7	13/15	86.7	2.1
	6–10	5/12	41.7	1.3	3/8	37.5	1.3	6/12	50.	1.7
	11–15	2/16	12.5	1.0	1/8	12.5	0	0/ 5	0	0
	16–30	0/14	0	0	0/9	0	"	1/ 7	14.3	1.0
	31–45	1/14	7.1	1.0	0/8	"	"	—	—	—
	46–53	0/10	0	0	—	—	—	—	—	—
Lung	1– 5	14/14	100.	1.7	7/8	87.5	1.9	13/15	86.7	2.0
	6–10	2/12	16.7	1.0	2/8	25.	1.5	6/12	50.	1.7
	11–15	5/16	31.3	"	"	"	1.0	2/ 5	40.	1.0
	16–30	0/14	0	0	0/9	0	0	1/ 7	14.3	"
	31–45	"	"	"	0/8	"	"	—	—	—
	46–53	0/10	"	"	—	—	—	—	—	—
Kidney	1– 5	10/14	71.4	2.0	7/8	87.5	2.0	14/15	93.3	1.6
	6–10	10/12	83.3	3.1	6/8	75.	2.3	9/12	75.	1.9
	11–15	5/16	31.3	2.0	2/8	25.	1.5	3/ 5	60.	2.5
	16–30	6/14	42.8	1.7	1/9	11.1	1.0	3/ 7	42.8	2.0
	31–45	5/14	35.7	1.8	2/8	25.	1.5	—	—	—
	46–53	1/10	10.	1.0	—	—	—	—	—	—
Liver	1– 5	14/14	100.	2.4	8/8	100.	3.0	14/15	93.3	3.4
	6–10	11/12	91.7	2.6	7/8	87.5	1.9	10/12	83.3	3.0
	11–15	14/16	87.5	1.6	4/8	50.	1.3	3/ 5	60.	1.5
	16–30	3/14	21.4	"	2/9	22.2	1.0	3/ 7	42.8	1.3
	31–45	"	"	1.3	1/8	12.5	"	—	—	—
	46–53	0/10	0	0	—	—	—	—	—	—
Spleen	1– 5	14/14	100.	2.7	8/8	100.	3.3	15/15	100.	3.3
	6–10	11/12	91.7	3.2	"	"	2.5	11/12	91.7	2.5
	11–15	12/16	75.	1.8	5/8	62.5	1.8	3/ 5	60.	1.5
	16–30	5/14	35.7	1.6	3/9	33.3	1.7	5/ 7	71.4	2.0
	31–45	2/14	14.3	1.0	1/8	12.5	1.0	—	—	—
	46–53	1/10	10.	1.0	—	—	—	—	—	—

* I = Primary challenge with 35 million organisms. II = Challenge 6 wk after dose of 7 million heat-killed organisms. III = Challenge 6 wk after dose of 0.7 million organisms.

† Numerator = No. positive cultures. Denominator = Total No. cultured.

‡ Avg relative concentration of organisms (see text).

liver (3 to 1.9) and spleen (3.3 to 2.5) between 6 to 10 weeks, as compared to that observed between 1 to 5 weeks; this observation was in contrast to the results noted in Group I where average concentrations in the liver rose slightly from 2.4 to 2.6, and in the spleen from 2.7 to 3.2 (Table I).

Similar results were obtained in the mice which previously had survived sublethal infections with 0.7 million organisms (Group III, Table I). Here again as in Group II there seemed to be the trend downward at 6 to 10 weeks as compared to 1 to 5 weeks in the average concentration of organisms in both the liver and spleen from 3.4 to 3.0 and 3.3 to 2.5, respectively. In this group which had received 2 inoculations of viable organisms, the liver and spleen had 42.8 and 71.4% positive cultures, respectively, between 16 to 30 weeks. During this same time interval the liver had about 22% and the spleen about 35% positive cultures in both Groups I and II.

Discussion. Mortalities in white mice following intraperitoneal inoculation of yeast phase *H. capsulatum* in mucin usually occur within 30 days(1,2). Although deaths from specific infection rarely occur after this period it is evident that organisms are still present in the heart's blood and lungs as long as 11 to 15 weeks after inoculation. The spleen, liver and kidney show positive cultures as late as 45 weeks post-inoculation. Relatively little difference was noted between mice receiving primary challenge (Group I) as compared to those receiving heat-killed organisms

(Group II) or sub-lethal doses (Group III) 6 weeks prior to challenge. The only suggested differences were: 1) an almost 2-fold greater number of positive liver and spleen cultures between 16 to 30 weeks in Group III as compared to the other 2 groups; this could probably be attributed to the residual viable organisms from the inoculation made 6 weeks prior to challenge. 2) A relatively higher concentration of organisms in the liver and spleen between 6 to 10 weeks in Group I as compared to the other 2 groups. When compared to the concentrations of organisms observed between 1 to 5 weeks, there was a definite trend downward in the previously infected or "immunized" mice as compared to the relative rise at 6 to 10 weeks in the primary challenge group.

Summary. Yeast phase *H. capsulatum* when inoculated, intraperitoneally, into white Swiss male mice may be recovered frequently from reticuloendothelial tissues as late as 45 weeks after infection. The lungs and heart's blood usually clear within 16 weeks post-inoculation. Mice surviving previous sub-lethal challenge or those previously given 1 dose of heat-killed organisms show little variation in these respects, when challenged, from those receiving primary challenge.

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Inhibition of Immunological Enhancement by Endotoxin in Refractory Rabbits. Immunochemical Study.* (22280)

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When Gram negative bacterial endotoxins are repeatedly administered by vein over short periods of time, resistance to the reactions normally produced by these toxic complexes rapidly develops(1). Quantities of endotoxin originally productive of vigorous systemic adjustments no longer produce untoward reactions(2,3). For example, febrile responses normally produced are either obliterated completely(2) or modified. Hemorrhagic and necrotizing phenomena normally produced in tumor tissue and skin prepared for the local Shwartzman reaction do not occur(3,4) after intravenous injection of toxin in refractory rabbits. The generalized Shwartzman reaction is no longer produced by paired spaced intravenous injections of toxin, and the leukopenia producing action of endotoxin is obliterated(5). In addition, refractoriness to the lethal effect of endotoxins occurs(6). Refractoriness to most of these reactions develops very soon after intravenous injections of endotoxin are begun. Resistance to the lethal effect of toxin may be demonstrated as early as 24 hours following the first intravenous injection(7). Refractoriness to each of the other effects of endotoxin occurs within a few days(2-4). This development has been dissociated from the classical immune response primarily by its lack of specificity(8), rapidity of development(7), shortness of duration(2), and lack of correlation with appearance and level of circulating antibody(9,10). Further, agammaglobulinemic patients unable to produce demonstrable circulating antibody develop refractoriness to febrile and intoxicating actions of endotoxins just as readily as do normal persons(11). On

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the other hand, refractoriness is promptly reversed following intravenous injection of certain colloidal substances(8,12), an effect attributed to the capacity of these colloidal substances to produce "blockade" of the reticuloendothelial system(13). Our recent studies have shown that the reactions produced by administration of Gram negative bacterial endotoxins result in profound disturbances in defense mechanisms of the host(14). Meningococcal endotoxin in quantities which prepare rabbits for the generalized Shwartzman phenomenon decreases the ability of rabbits to clear colloidal materials from the blood presumably by interfering with the function of the reticuloendothelial system(13). Further, endotoxin seemed to enhance certain bacterial infections in rabbits. For example, when toxin was given prior to establishment of a pneumococcal skin infection, bacteremia and death resulted whereas in control rabbits given no endotoxin the organism produced only local skin infection(14). In contradistinction, endotoxin did not enhance susceptibility to infection when injected intravenously in rabbits previously made refractory to the endotoxin(14). Indeed, refractory rabbits appeared to resist pneumococcal and streptococcal infection more vigorously than did controls. These observations provoked investigation of the effect of meningococcal endotoxin on the immune response in rabbits as revealed by immunochemical study. In confirmation of observations of Johnson *et al.* (15) we found that when toxin was given together with bovine serum albumin, enhancement of antibody production against the foreign serum protein resulted(16).

The present study was undertaken to determine whether refractoriness to the febrile, toxic, and necrotizing effects of endotoxin would interfere with the adjuvant effect of this material on the immune response.

Materials and methods. Animals employed were 2 kg albino rabbits obtained from one breeder and maintained throughout the experiment on a diet of Purina rabbit pellets. Endotoxin was prepared from lots of a culture-free meningococcal[‡] filtrate of high Shwartzman activity. Different lots were combined, spun at 15,000 rpm, the supernatant quick-frozen and stored at –30°C. Details of preparation have been described previously(17). The refractory state was induced using a regimen which had proved to be effective in quickly producing a high degree of resistance in a short time, including refractoriness to fever, lethal effect, necrotizing action and production of leukopenia. This schedule consisted of 11 daily intravenous injections of 2 cc of endotoxin in increasing amounts, beginning with a dilution of 1:10,000 and extending to a dilution of 1:10 on the 11th day. Toxin solutions were prepared daily from the frozen culture free filtrates and dilutions made up with pyrogen free saline. Attempt to provoke the Shwartzman reaction was used as the test for resistance since it had previously been shown that this was a delicate index of susceptibility to the action of endotoxin. This reaction was ordinarily produced by an intradermal preparatory injection of 0.25 ml of 1:2 dilution of endotoxin, followed in 18 hours by an intravenous injection of the same material. In the stock of rabbits used and with the endotoxin employed, the Shwartzman reaction developed in an incidence between 95 and 100%. All injections, with the exception of the intradermal preparation for the local Shwartzman reaction, were made by way of the marginal ear vein. Crystallized bovine serum albumin (BSA), lot #67009 (Armour and Co., Chicago, Ill.), was employed as the antigen. Immediately prior to injection, solutions containing 15 mg BSA were made up to 2 ml with either pyrogen free saline or 1:100 dilution of the endotoxin depending upon the experimental group. Prior experiments established that this quantity of toxin represents the optimal dose for producing enhancement of antibody formation with the quantities of antigen used. Rabbits were divided into 3 groups; Group I normal, non-refractory rabbits given BSA together with endotoxin intravenously, Group II refractory rabbits given endotoxin and BSA antigen intravenously, and Group III normal rabbits given BSA intravenously.

[‡] Strain 44-B, generously supplied by Dr. Gregory Shwartzman, Mount Sinai Hospital, New York.

Blood was collected by cardiac puncture on the 11th day following antigenic stimulation, since previous studies had established that at this time circulating antibody to BSA is at its highest level. Clots were broken up after standing 24 hours at 4°C, the tubes centrifuged for 20 minutes at 1000 RPM, the serum decanted into lusteroid tubes, frozen at –70°C, and stored at –30°C until antibody determinations were performed.

Serum antibody content was determined using a modification of the quantitative precipitin method described by Kabat and Mayer(18). The method involves two precipitations. The first, a preliminary run, requires only small quantities of serum and is used primarily to determine the optimal antigen concentration to be used in the final precipitation. With the preliminary precipitation, a surprisingly accurate estimate of antibody content can be made merely by analysis of the supernatant for the presence of excess antigen. Known concentrations of antigen were prepared from standard stock solution of BSA prior to each precipitation. Serum samples were thawed at 37°C for 10 minutes, mixed and centrifuged in the cold (4°C) for 1 hour at 3000 rpm. Aliquots of each serum sample were then precipitated with 4 concentrations of antigen prepared from the standard BSA solution. Precipitin tubes were incubated at 37°C for 1 hour and stored in the cold 7 days to insure complete precipitation with agitation each day. Tubes were then centrifuged at 4°C for 1 hour at 3000 rpm and supernates saved for analysis. Excess antigen was determined by capillary tube precipitation with a potent anti-BSA rabbit serum. The tubes were incubated 1 hour at 37°C, placed in the cold and read for precipitate at 36 hours. Final determinations were performed in a similar manner except that nitrogen content of the precipitates was determined. Precipitation was carried out with antigen concentrations known to fall in the equivalence zone of each serum sample. Precipitates were washed twice with 0.145 molar saline (4°C) and precipitate nitrogen was determined colorimetrically using Heidelberger MacPherson modification of the Folin-Ciocalteau reaction(19). Optical density, read at 650 mμ on a Coleman Universal Spec-

TABLE I.

Group	Experimental procedure	Serum antibody content		
		γ N/ml	t	P
I	2 ml I.V. solution containing 15 mg BSA + 1:100 dilution of meningococcal endotoxin	333		
		201		
		74		
		121		
		94		
		233		
	Mean	176		
II	Resistant rabbits, 2 ml I.V. solution containing 15 mg BSA + 1:100 dilution meningococcal endotoxin	45		
		58		
		<10		
		<10		
		58		
		<10		
	Mean	31.8		
III	Controls—2 ml I.V. solution containing 15 mg BSA only	47		
		<10		
		<10		
		28		
		35		
	Mean	26.0		
I & II			3.47	<.02
I & III			3.65	"
II & III			.47	>.50

trophotometer Model 14, was converted to mg nitrogen using a standard calibration curve prepared with a highly purified human gamma globulin.§ Nitrogen content of the stock solution was determined by the micro-Kjeldahl method.

Results. Resistance was developed to a high degree in rabbits receiving daily intravenous injections of meningococcal endotoxin. Six of the 7 rabbits of Group II showed no evidence of a positive local Shwartzman reaction. Lack of usual febrile responses to a standardized pyrogenic dose of endotoxin was characteristic of the resistant rabbit.

The results of the quantitative precipitin determinations are shown in Table I. When meningococcal endotoxin was given together with BSA, a significant enhancement of antibody formation resulted. The mean level of circulating antibody to the BSA on the 11th day following antigenic stimulation was 6 times that of the control animals of Group III which received BSA alone. The probability that this might result from chance distribution is <0.02 which indicates that there exists a highly significant difference between these groups. The quantity of endotoxin employed, 2 ml of 1:100 dilution, was in that range recently reported to be optimal for producing an adjuvant effect resulting in increased levels of circulating antibody against apparently unrelated foreign serum protein(16).

Resistant animals of Group II received the same antigenic stimulation that resulted in greatly elevated levels of circulating antibody in the rabbits of Group I. However, serum antibody levels of the resistant animals on the 11th day following antigenic stimulation were not elevated. The difference between Groups I and II were also significant while statistical analysis revealed no difference between Groups II and III.

Discussion. It is shown in these experiments that resistance to the adjuvant effect of endotoxin was readily induced by repeated daily intravenous injections of increasing amounts of this material. A solution consisting of bovine serum albumin and meningococcal endotoxin produced significantly more antibody when given to normal untreated rabbits then when injected intravenously into refractory animals.

In these experiments the capacity of endotoxin to enhance antibody production is again demonstrated and it is further shown that this effect is prevented by prior treatment of rabbits with endotoxin according to a regimen designed to produce refractoriness to the other known effects of the toxin. These observations provide evidence that the enhancing effect of meningococcal endotoxin on the production of immune bodies cannot be explained simply as the synergistic effect of two unrelated antigens or by an unsuspected antigenic relationship between BSA and meningococcal endotoxin resulting in enhanced antibody production against the former.

It appears that expression of the "toxic" effect of endotoxin is necessary for the stimulation of antibody production by endotoxin observed in this and previous studies(15,16). The nature of this adjuvant effect remains enigmatic. Such an action could be directed specifically at the mechanism of antibody synthesis at a cellular or subcellular level or might act indirectly by mechanisms involving alteration in vascular or cellular permeability, circulatory dynamics, phagocytic function or central nervous system function. Whatever be the mode of action, the adjuvant effect of endotoxin appears to be real and to depend on the "toxic" properties of the bacterial product.

Summary and conclusion. 1. Using an immunochemical system, inhibition of the adjuvant effect of endotoxin by development of refractoriness to the "toxic" properties of endotoxin is demonstrated. 2. The basis for enhancement of immune body formation by endotoxin is discussed.

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§ Generously supplied by Dr. John T. Edsall, Harvard University, Boston.

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Progestational Effectiveness of 19-nor-Ethinyl-Testosterone* by Oral Route in Women. (22281)

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Tullner and Hertz(1) have previously demonstrated that 19-nor-progesterone is 4 to 8 times as active as progesterone by injection in the Clauberg rabbit test. Hertz, Tullner, and Raffelt(2) also showed that 19-nor-ethinyl-testosterone is likewise about 5 times as active as ethinyl testosterone when tested for progestational activity by oral administration in the Clauberg rabbit and that it is also highly active in the ovariectomized monkey pretreated with estrogen(2,3).

This report presents observations on the progestational effectiveness of 19-nor-ethinyl-testosterone[†] upon oral administration in 4 women.

Case 1: S.J.M. 14-year-old white female; proven by biopsy of ovarian anlagen to have ovarian agenesis (October, 1953). She was maintained on cyclic estrogen therapy with monthly withdrawal bleeding until January, 1954. Then after 23 days on 0.1 mg ethinyl estradiol daily she received 10 mg 19-nor-ethinyl-testosterone[†] twice daily for 12 days. Suction curettement of the endometrium showed marked progestational effects with numerous polyhedral as well as spindle cells in the stroma. Menses ensued 2 days after cessation of therapy.

Case 2: A. H. 21-year-old white female; ovarian agenesis proven by biopsy of both ovarian anlagen in November, 1953. She was maintained on cyclic estrogen therapy until January, 1954, when she was given 5 mg of 19-nor-ethisterone twice daily in addition to her usual daily estrogen dosage of 0.1 mg ethinyl estradiol for 2 days followed by 12 days more of the nor-ethisterone alone. She began menstruating at this point and all treatment was discontinued for 3 months. This bleeding indicated that although 5 mg of nor-ethisterone twice daily was sufficient to markedly prolong her usual latent period for withdrawal bleeding, nevertheless it was not sufficient to maintain the endometrium. Accordingly, about 2 weeks later another course of estrogen followed by 10 mg of nor-ethisterone twice daily was given for 14 days and a suction curettement performed. The endometrium showed a marked progestational effect with distinct decidual reaction in many areas.

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* We are greatly indebted to Chemical Specialties Co., N. Y., for supplies of 19-nor-ethinyl-testosterone.

[†] Also called: "nor-ethisterone."

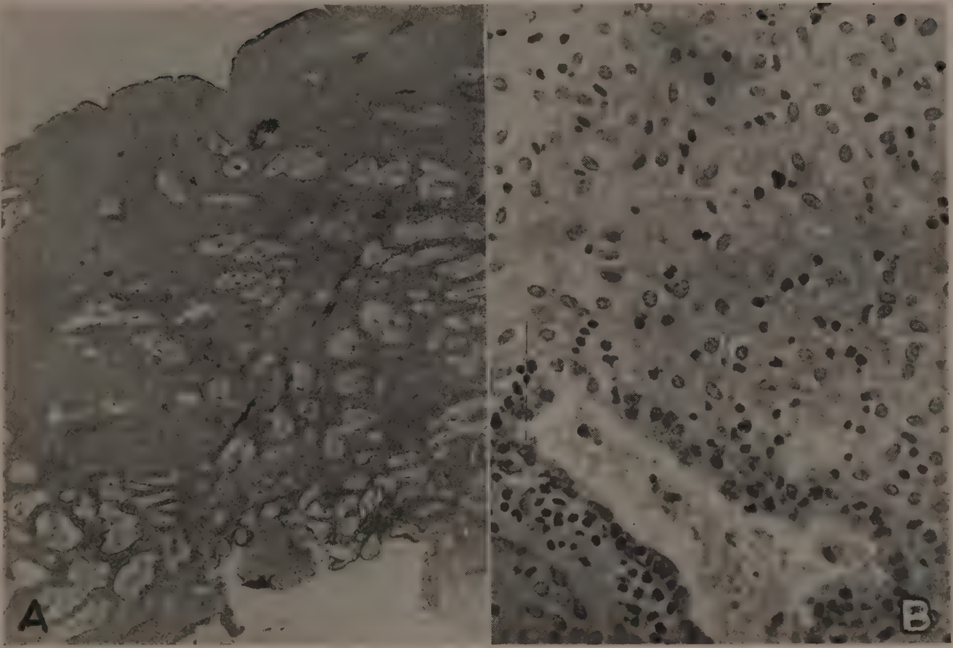

FIG. 1A. Curettage specimen from case 4 after 25 days on 19-nor-ethisterone at 10 mg twice daily.

FIG. 1B. Decidual cells in case 4.

Case 3: M. H. 24-year-old white married female; amenorrhoea of 4 years duration. Menses had been entirely normal (interval 28 days; flow 5 days; napkins 3-4) since 13 years of age. She was given 5 mg of nor-ethisterone twice daily for 5 days and no menses occurred on withdrawal. A second course of 10 mg nor-ethisterone twice daily for 5 days was followed after 3 days by menses lasting about 5 days. Since no spontaneous menstruation occurred during the ensuing 35 days she was given another course of 10 mg of nor-ethisterone twice daily for 5 days and this was followed by menses. Six weeks later she was given ethinyl testosterone at a dose of 40 mg twice daily for 5 days. No bleeding had ensued when the patient was seen 41 days later and further followup was not obtainable.

Although no histological evidence was obtained in this case, 10 mg nor-ethisterone twice daily elicited menses on 2 occasions and half this dose failed to do so. In addition, a 4 times greater dose of ethinyl testosterone also proved ineffective.

Case 4: C.P. 40-year-old white female; carcinoma-in-situ diagnosed by repeated smears and biopsy. She was placed on 10 mg nor-ethisterone twice daily continuously for 25 days while awaiting hysterectomy. No menses intervened during this period. Papanicolaou smears throughout this period remained positive. Quadrant biopsies of the cervix prior to hysterectomy revealed invasive carcinoma of the cervix. Endometrial curettement showed extreme progestational effect with extensive decidual reaction (Fig. 1). Panhysterectomy with node dissection was then carried out.

Conclusions. It is clear from the foregoing observations that 10 mg nor-ethisterone given twice daily represents a reproducibly effective dose in women for the production of marked progestational changes in the endometrium. The data suggest that very probably one half this dose will not be effective. The comparably effective dose of ethinyl testosterone (ethisterone) would be about 4 times higher(4). Thus the comparative values agree reasonably well in rabbit, monkey, and man(2,3).

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Cytological and Weight Changes in Pituitary Gland of the Severely Stressed Rat.* (22282)

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Numerous cytological studies have been made of the anterior pituitary gland following the application of different types of stress. Finerty and Briseno-Castrejon(1) and Brokaw *et al.*(2) reported an increase in the percentage of acidophils as shown by azocarmine after unilateral adrenalectomy in rats. Daughaday, Perry and McBryde(3) associated the acidophils with ACTH secretion in their studies of increased adrenal function in acromegaly. Finerty, Hess and Binhammer (4) reported no increased percentage of acidophils or basophils at intervals of 1, 3, 12 and 24 hours after stress with the azocarmine staining method but did describe an increase in numbers of acid hematein positive cells. No change in the percentage of the basophils by staining with PAS or with aldehyde fuchsin was found. Recently, Knigge(5) reported an increase in percentage of acidophils and argyrophilic basophils 12 hours after severe stress, and concluded that the latter were most probably associated with ACTH production since response to stress occurs in thyroidectomized animals which are characterized by absence of acidophils. Kraus(6) reported a decrease in the number of basophils in the anterior pituitary of Addison's disease patients. Further evidence for the basophils as the cytological source of ACTH was noted by Koneff(7) who found a decrease in the basophils after bilateral adrenalectomy and after ACTH injection in rats. D'Angelo *et al.*(8) noted adrenal hypertrophy during prolonged periods of inanition in the guinea pig. This was accompanied by loss of weight of the pituitary and hypertrophy and hyperplasia of the basophils, increasing with the severity of the inanition. Marshall(9) found ACTH antibody coupled with fluorescent dye to be localized in the basophil cells of the hog pituitary. Recently, Wilson *et al.*(10) reported the characteristic Crooke's hyaline cytoplasmic change of the pituitary basophils associated with hyperfunction of the adrenal cortex, and a progressive loss of acidophils in 100 routine human necropsies.

It was the purpose of the present experiments to reinvestigate histological changes and to study changes in weight of the pituitary gland in order to gain further insight into the effects of a severe stress.

Method. Male rats of the Holtzman strain weighing between 110-130 g were used. They were stressed by immersion (except for the head) in water (containing Aerosol) at 70°C for 5 seconds while under Nembutal anesthesia. All animals were sacrificed by decapitation. The pituitary gland was removed immediately and weighed on a Roller-Smith torsion balance. About half of the glands were then placed in appropriate fixative for future histological study. The rest were placed on previously weighed coverslips, dried in an oven at 30°C for 48 hours and re-

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* These studies were aided by contract between Office of Naval Research, Department of the Navy, and University of Texas.

TABLE I. Number of Cells per Field* in Anterior Pituitary after Severe Stress Using Various Staining Methods.

Hr after stress	No. of animals	Acid hematein	Azocarmine			Aldehyde fuchsin		PAS
			Acid.	Baso.	Chrom.	Beta	Delta	
Unstressed	30	21.1 ± .5†	15.7 ± .6	7.0 ± .2	41.9 ± .5	4.6 ± .5	3.6 ± .4	7.8 ± .6
1	35	18.5 ± .5	16.6 ± 1.0	7.7 ± .4	33.5 ± .9	4.2 ± .3	4.4 ± .2	8.0 ± .4
3	32	20.0 ± .8	17.3 ± 1.3	9.8 ± .7	39.3 ± 1.6	3.7 ± .4	5.2 ± .3	8.3 ± .4
12	32	29.4 ± .8	25.5 ± .6	9.9 ± 1.0	51.4 ± 2.5	3.1 ± .4	6.4 ± .5	9.9 ± .5

* Oil immersion 1.25 mm lens and a 20× eyepiece.
† ± stand. error.

weighed. Glands used for histological study were stained by 5 different methods: fixation in Bouin's fluid and stained with hematoxylin for nuclei counts; fixation in Zenker-formol solution and staining with a modified azocarmine stain(11) for cell counts; fixation in formol-sublimate and staining with periodic acid Schiff method for basophils(12); fixation in formol-calcium and staining with acid hematein for phospholipids(13); and fixation in Susa-picric acid and staining with aldehyde fuchsin to distinguish beta and delta cells(14). Cell counts were made on 3 serially cut sections selected at approximately the ¼, ½ and ¾ levels of the gland. The cells were counted in every tenth field for 10 consecutive fields. A standard field was obtained with a 1.25 mm oil immersion Spencer lens and 20x eyepiece. Cell counts were recorded as the number of cells per field.

Results. Exp. I. Ninety-nine pituitaries were prepared for histological study from rats which were sacrificed at intervals of 1, 3 or 12 hours post-stress. Thirty unstressed animals served as controls. Table I shows no significant differences in number of anterior pituitary cells per standardized microscopic field among control, 1 and 3 hour post-stress animals by any of the staining methods. However, 12 hour post-stress animals demonstrated a significant increase in acid hematein positive cells per field. There also were significant changes in the number of acidophils, basophils and chromophobes per standard microscopic field in the 12 hour post-stress animals stained by azocarmine stain as compared to controls. PAS staining of pituitaries of the 12 hour post-stress animals showed an increase in number of basophils per field. The 12 hour post-stress pituitaries showed an increased number of delta cells and a slight decrease in beta cells when stained by Halmi's aldehyde fuchsin method (14). In the 12 hour post-stress groups all cell counts (except beta cells) after various methods of staining show a significant increase in number of cells per field as shown in Tables I and II.

Calculations of percentage of cells stained by the azocarmine method show that there is no significant change in percentage of each type of cell after severe stress. The nuclei counts are similar to the total cell counts after azocarmine staining as shown in Table II. Table I shows total number of basophils after staining by aldehyde fuchsin, azocarmine or PAS to be similar also. The acid hematein stain for acidophils gives consistently higher numbers than for acidophils

TABLE II. Relation of Fresh Pituitary Weight to Total Cells per Field* after Severe Stress.

Hr after stress	No. of animals	Body wt, g	Fresh pituitary wt		Total nuclei/field*	
			Absolute, mg	Relative, mg/100 g	Hematoxylin	Azan
Unstressed	30	119 ± 1.2†	4.11 ± .03	3.44 ± .03	65.6 ± .7	64.7 ± .9
1	35	118 ± 1.2	4.06 ± .02	3.44 ± .02	60.1 ± 1.4	57.4 ± .8
3	32	111 ± 1.6	3.71 ± .03	3.40 ± .02	61.9 ± .9	66.5 ± 1.2
12	32	112 ± 2.0	3.40 ± .03	3.05 ± .02	84.6 ± 2.1	87.2 ± 1.4

* Oil immersion 1.25 mm lens and a 20× eyepiece.
† ± stand. error.

TABLE III. Fresh and Dry Weight of Pituitary after Severe Stress.

			Rel. wt/100 g body wt		
Hr after stress	No. of animals	Body wt, g	Fresh, mg	Dry, mg	Liquid lost in drying, mg
Unstressed	31	121 ± 1.2*	3.41 ± .03	.75 ± .009	2.66 ± .01
1	11	122 ± 1.6	3.46 ± .04	.77 ± .01	2.69 ± .02
12	28	128 ± 2.4	3.01 ± .04	.62 ± .01	2.39 ± .02
Diff. between unstressed and 12 hr post-stress			.40	.13	.27
Relative fluid and solid loss (in %)				32.5	67.5

* ± stand. error.

stained by the azocarmine method as shown in Table I.

General observations of the 12 hour post-stress pituitaries indicated few mitotic figures, and an apparent reduction of cytoplasm in the cells, resulting in an increased nuclear-cytoplasmic ratio.

Results in Table II show no significant change in fresh weight of the pituitary of 1 and 3 hour post-stress animals as compared with controls. There is also no significant change in relative fresh weight per 100 g body weight of 1 and 3 hour post-stress animals. The 12 hour post-stress pituitaries show a significant absolute decrease in weight which is also apparent relative to body weight.

Exp. II. Thirty-nine male rats were stressed and sacrificed at 1 and 12 hours later. Pituitaries were removed, weighed and dried. Determinations of relative weight per 100 g body weight were made of fresh weight, dry weight and weight of fluid lost in drying. The same determinations were made on 31 control animals.

Table III shows no significant changes from controls in the relative fresh weight, relative dry weight and relative weight of fluid lost in the 1 hour post-stress animals. In the 12 hour post-stress animals there was a decrease in relative fresh weight of the pituitary per 100 g body weight similar to that in Exp. I. The relative dry weight and relative weight of fluid lost in drying of the 12 hour post-stress animals show a decrease compared to controls. Table III also shows that 32.5% of the difference in weight between the controls and 12 hour post-stress animals is due to loss in solids and 67.5% of the difference is due to a loss in fluid.

To determine the effects of a severe stress on the weight of an organ of similar anatomical position which has little endocrine function, the brains of controls and 12 hour post-stress animals were removed, weighed, dried and reweighed. No change in fresh or dry weight of the brain was found as a result of severe burns suggesting that the two organs respond differently.

Discussion. Our results confirm previous reports of increase in number of acid hematein-positive cells after stress with no change in percentage of azocarmine staining acidophils(4). This severe burn stress after 12 hours induces some increase in number of all cells per microscopic field, regardless of staining method. This is probably a result of general shrinkage of the pituitary gland, as indicated by weight reduction and increased nuclear-cytoplasmic ratio. Such a finding points out a variable factor which must be considered in interpretation of pituitary cytological studies whenever there is a possibility of pituitary anhydremia.

Measurements of water content of pituitaries after stress indicate that about 68% of the weight reduction at 12 hours post-burn is a result of dehydration. This is compatible with hemoconcentration usually seen in burned patients due to edema and resulting anhydremia. Since the same fluid changes were not found in the brain it is concluded that the pituitary is one of the organs specifically affected by anhydremia, possibly because of its oral epithelial origin.

About 32% of pituitary weight reduction at 12 hours post-burn is a result of loss of solids. Since solids make up only one-fifth of total weight of the gland, it is apparent that their loss is a significant aspect of the response to stress, amounting to an actual loss of 17% of all solids present. Possible factors responsible for the loss of solids may be loss of diffusible cellular proteins, loss of mineral ions to the blood, or excess secretion of pituitary hormones.

Summary. Effects of severe stress, immersion in water at 70°C for 5 seconds, were studied on the male albino rat in relation to response of the anterior pituitary. Using 5 different staining methods, no significant changes in *percentage* of acidophils, basophils or chromophobes were found 1, 3 or 12 hours after stress, although there were marked increases in *number of cells per field* in the 12 hour post-stress rats. Fresh weight of the pituitary showed no significant change in 1 and 3 hour post-stress animals, but the 12 hour group displayed a marked reduction, which was evident on both an absolute and a relative to body weight basis. Comparison of relative dry weight and amount of fluid lost in drying in unstressed and 12 hour stressed animals revealed that the weight decrease consisted of both fluid loss and reduction in solids.

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Hemolysis of Human Erythrocytes by a Sulfhydryl Inhibitor, p-Chloromercuribenzoic Acid.* (22283)

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Although sulfhydryl groups have a function in the metabolism and integrity of erythrocytes, its nature is poorly defined. Ingbar (1) reported that cysteine caused sickling of erythrocytes from patients with sickle cell anemia. It was prevented by the addition of heavy metals or p-chloromercuribenzoic acid. Benesch and Benesch(2) have shown that either phenylmercuric hydroxide or mersalyl (salyrgan) caused hemolysis of sheep erythrocytes in a fairly consistent pattern; and that this hemolysis was blocked by sulfhydryl groups but not by sulfur in the disulfide linkage or other forms of sulfur. Dimant, *et al.* (3) more recently have been concerned with the question of *de novo* synthesis of glutathione by erythrocytes. In our previous studies(4) on the feeding of methionine, prothrombin and accelerator globulin decreased in all 3 human subjects. Increased erythrocyte destruction was demonstrated in only one subject.

This present study concerns the relation of certain factors of the red cell environment to hemolysis caused by p-chloromercuribenzoic acid.

Method. The sodium salt of p-chloromer-

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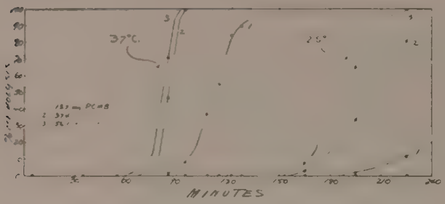

FIG. 1. Effect of concentration and temperature on hemolysis by the sodium salt of p-chloromercuribenzoic acid. Erythrocytes were added at 0 min. There was no hemolysis in the saline control indicated by hemispheres on the base line.

curibenzoic acid† (PCMB) was prepared in 5x10^{-4} molar concentration in 0.9% sodium chloride solution. Erythrocytes were prepared from heparinized blood of normal human donors. The cells were washed 3 times in cool 0.9% sodium chloride solution. After the last washing 0.5 ml of packed cells was added to 25 ml of 0.9% normal saline containing the chemicals studied in the particular experiment. Unless otherwise specified, the cells were incubated at 37°C during the period of observation. The degree of hemolysis was determined by measuring the amount of hemoglobin in the supernatant fluid of a suspension of cells at intervals throughout the study. By relating this figure to 100% hemolysis, obtained by completely hemolyzing a homogeneous suspension of the cells and measuring the optical density in a photometer, the per cent of hemolysis at any time could be calculated. Any existing hemolysis was measured. Correction was made by subtracting this value from the completely hemolyzed sample or the samples obtained at various times. The following equation shows this relationship:

$$\%\ \text{hemolysis} = \frac{OD_t - OD_c}{OD_m - OD_c} \times 100$$

OD_c = Optical density of supernatant immediately after adding red cells.

OD_m = Maximum optical density possible with this concentration of red cells (100% hemolysis).

OD_t = Optical density of supernatant at any time during test.

Zero time was the instant of adding the red cells. The samples of hemoglobin to be measured in the photometer were in a weak solution of ammonium hydroxide. No effort was made to control the pH. During hemolysis the pH of the supernatant fell from 7.6 to 7.3.

Results. 1. *Effect of PCMB concentration*

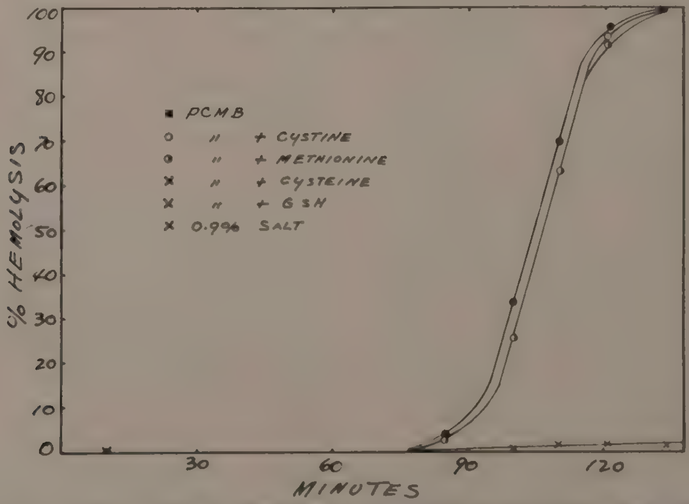

FIG. 2. Amino acids containing sulfhydryl groups inhibit the hemolytic effect of PCMB; those without sulfhydryl are ineffective. There is very little hemolysis of the control erythrocytes in 0.9% saline suspension in 4 hr. Erythrocytes were added at 0 min.

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† Purchased from Sigma Chemical Co.

and temperature on hemolysis (*Fig. 1*). Increasing the concentration of PCMB 2 and 3 times the original 5x10^{-4} molar resulted in a slight increased rate of hemolysis; and the onset of hemolysis after initial contact with the reagent began at 75 instead of 90 minutes. Decreasing the concentrations of PCMB progressively prolonged the hemolytic process over a period of 3 to 4 hours. Under standard conditions with a concentration of PCMB of 5x10^{-4} molar at 37°C hemolysis began in about 90 minutes and was completed in 120 to 130 minutes. This has been a fairly consistent observation with different samples of PCMB and erythrocytes from different donors. Incubation at 25°C lengthened the time before the onset of hemolysis and, once hemolysis had begun, the rate was slower.

II. *Inhibition of hemolysis by amino acids containing –SH groups added to PCMB solution before contact with erythrocytes* (*Fig. 2*). Amino acids containing sulfhydryl groups in equimolar concentrations inhibited the hemolytic effect of PCMB if added to the solution before the erythrocytes. Cystine and methionine were completely ineffective in this inhibition.

III. *Effect of addition of –SH groups after contact of PCMB and erythrocytes* (*Fig. 3*). An equimolar concentration of glutathione was added immediately after the red cells were suspended in the PCMB solution and 30, 60 and 90 minutes later. At one minute and 30 minutes most hemolysis was prevented. There was considerable inhibition of the PCMB effect when glutathione was added as late as 60 minutes. Even at 90 minutes, just before hemolysis was about to begin, the addition of glutathione altered the curve remarkably although hemolysis went to completion.

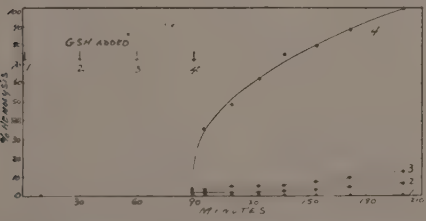

FIG. 3. Inhibition of hemolysis when glutathione added at intervals after contact between erythrocytes and PCMB. Erythrocytes were added at 0 min.

IV. *Effect on hemolysis of washing erythrocytes after contact with PCMB* (*Fig. 4*). Erythrocytes were washed 3 times in cool saline after one minute in contact with PCMB and after 30 and 60 minutes. The washed cells were suspended in 25 ml of fresh saline. Washing the erythrocytes one minute after contact with PCMB solution virtually eliminated the hemolytic effect. Washing after 30 minutes reduced the rate of hemolysis so that there was 20 per cent hemolysis at 210 minutes instead of the usual 90 minutes. Washing at 60 minutes had little influence on the onset and rate of hemolysis except that about 10% of the cells were resistant to hemolysis for over 4 hours. This finding was in contrast to that of the previous experiment when the addition of glutathione at 60 minutes prevented most hemolysis. The supernatant from the initial separation in this study was saved and tested for hemolytic activity by adding another 0.5 ml of red cells. These curves were reciprocals of those in Fig. 4, indicating a progressive loss of hemolytic activity from the solution the longer the erythrocytes had remained in it. The supernatant removed at 60 minutes was free of hemolytic effect. However, contact for 1 minute produced no detectable loss in hemolytic activity. The reaction between erythrocytes and PCMB is not instantaneous but requires a lapse of time.

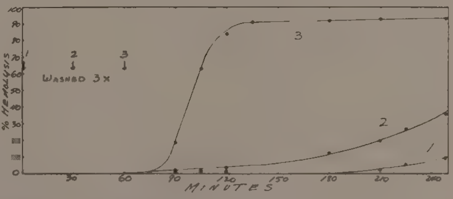

FIG. 4. Incomplete inhibition of hemolysis by washing PCMB from erythrocytes. Red cells were added to PCMB at 0 min.

V. *Exhaustion of PCMB hemolytic effect by exposure of PCMB to single aliquot of red cells* (*Fig. 5*). The usual suspension of red cells in PCMB solution was made and

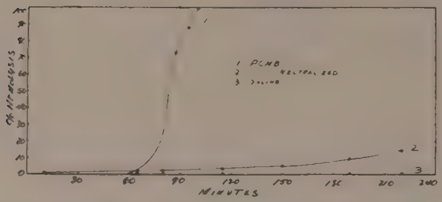

FIG. 5. Exhaustion of hemolytic effect of PCMB by one exposure to erythrocytes. In this chart only, time scale is measured from time when red cells were added to the PCMB in (1) and time when a second portion of red cells was added to the hemolyzed mixture in (2), time when the cell suspension was made in saline in (3).

hemolysis was allowed to proceed. As soon as hemolysis was complete another 0.5 ml of red cells was added to this hemolyzed mixture and more observations were made. There was very little hemolysis of the second portion of red cells, which indicates that the hemolytic effect of PCMB was exhausted by the first aliquot of red cells. This then raised the question as to whether the exhaustion of the hemolytic effect was the result of –SH groups in the cell stroma or inside the cell envelope.

VI. *Exhaustion of PCMB hemolytic effect by hemoglobin but not by stroma (Fig. 6).* A suspension of stroma and a hemoglobin solution were prepared by thrice rapidly thawing and freezing 0.5 ml of packed cells. A few milliliters of cold 0.9% sodium chloride solution were added to the hemolyzed mixture which was then centrifuged. The clear supernatant was transferred to a tube and the quantity was made up to 25 ml with 0.9% sodium chloride solution. A small quantity of fluid containing the stroma was placed in another tube and the volume made up to 25 ml with 0.9% sodium chloride solution. Microscopic examination showed the hemoglobin portion to be virtually free of stroma and the stroma to be concentrated in the other tube. PCMB was added to the two tubes in the concentration of 5x10^{-4} molar followed by incubation for 90 minutes. Then 0.5 ml of erythrocytes was added to each tube and the usual procedure followed. The stroma fraction altered the shape of the curve but only to an insignificant degree. Hemolysis in the other tube was almost completely inhibited by the hemoglobin solution. Since the stroma was not washed the alteration in this curve may have been caused by the small amount of free hemoglobin in the stroma suspension.

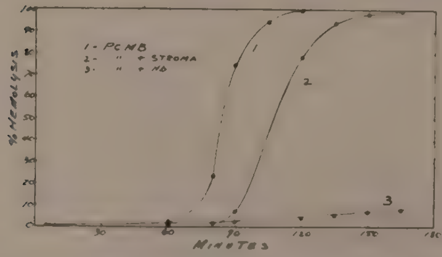

FIG. 6. Inhibition of PCMB hemolytic effect by incubation with hemoglobin solution and failure of inhibition by stroma suspension. Test erythrocytes were added at 0 min.

VII. *Effect of glutathione plus glucose on the PCMB hemolytic system (Fig. 7).* Glutathione in equimolar concentration with PCMB and glucose to produce a final concentration of 1 mg per 1 ml was added at intervals of 1 minute, 30 minutes and 60 minutes to the red cells suspended in PCMB solution. Glucose had no beneficial effect and was harmful when added at 60 minutes.

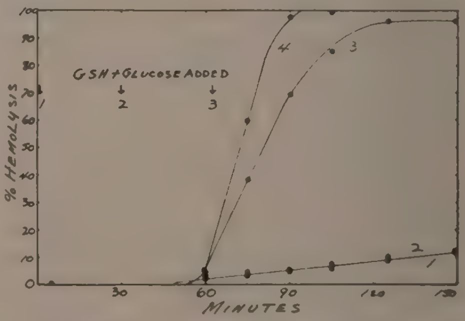

FIG. 7. Failure of glucose to modify inhibition of PCMB hemolysis by glutathione. Erythrocytes combined with PCMB at 0 min. In (4) red cells were suspended in solution of PCMB and NaCl only.

Discussion. Organic mercurials which combine with –SH groups cause hemolysis of human erythrocytes. Previous transformation of the organic mercury compound into the mercaptide with cysteine or glutathione inhibits hemolytic activity. The rate of hemolysis is influenced by temperature(11) as well as concentration of the mercurial reagent. Benesch *et al.*(5) found a striking variation in the reactivity of the –SH groups of crystalline hemoglobin among different species. Those of dogs and man were less reactive than those of sheep. Racker(6) suggested that the difference in rate of reactivity between sulfhydryl groups and reagents may be influenced by properties of adjacent groups on the protein molecule.

That –SH groups are necessary for the integrity of the erythrocyte is a fact. The thiols responsible for this effect are not known nor is the mechanism known. A recent report (5) indicates there are 8 moles of –SH per mole of hemoglobin in man. Previous studies have demonstrated the presence of ergothioneine(7-9) and glutathione(10) within the erythrocyte. The glutathione is in a dynamic state(3) and may traverse the cell envelope. Less is known of the role of ergothioneine.

The necessity of –SH groups for the integrity of the erythrocyte suggests that control of the thiol system may be advantageous in the preservation of whole blood stored for transfusion.

Conclusions. 1. The sulfhydryl reagent, p-chloromercuribenzoic acid, causes hemolysis of human erythrocytes. This action can be prevented by prior reaction with sulfhydryl groups. 2. The hemolytic action of PCMB is reversible by washing cells for as long as 30 minutes after contact with this agent. Reversibility of hemolysis lasts up to 60 minutes after addition of free sulfhydryl group. 3. No protection from PCMB hemolysis was noted with stroma whereas hemoglobin solution was effective. 4. The reaction between PCMB and the –SH group of erythrocytes is relatively slow. 5. A physiologic concentration of glucose offered no protection from hemolysis in this system.

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Pyridoxine Responsive Anemia in the Human Adult.* (22284)

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Although pyridoxine is necessary for normal hematopoiesis in dogs(1,2), swine(3-5) and monkeys(6), no evidence is available that abnormal hematopoiesis occurs in humans secondary to a naturally occurring deficiency of pyridoxine or alteration in its metabolism(7,8). In general, the hematologic alterations resulting from pyridoxine deprivation in animals have been characterized by a hypochromic, microcytic anemia with high plasma iron; although a moderate leukopenia is usually observed, neutrophilic leukocytosis has also been described. After pyridoxine deprivation abnormal metabolites of tryptophan are excreted following a loading test dose of this amino acid(9,10). The administration of pyridoxine is followed by a well-defined reticulocyte response, rapid fall of plasma iron and restoration of normal leukocyte, erythrocyte and hemoglobin values; concomitantly, the abnormalities of tryptophan metabolism are corrected.

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The following observations concern a 35-year-old white male who had a severe hypochromic anemia unresponsive to the usual hematopoietic agents and associated with abnormalities in tryptophan and iron metabolism; these abnormalities were promptly reverted to normal following the intramuscular administration of pyridoxine hydrochloride.

Results. Patient H.S. was first admitted to the hospital in 1947 at 27 years of age, because of weakness and pallor. Except for nocturia, tibial pain and pedal edema that became very bothersome in 1955, the patient's signs and symptoms during entire subsequent course could be directly attributed to decreased hemoglobin level and were abolished by transfusions. No signs or symptoms of glossal, neural, or dermal involvement occurred. His appetite and food intake always remained excellent and he maintained a constant weight. Detailed dietary history showed that the daily caloric intake averaged 2900 and was comprised of 122 g protein, 284 g carbohydrate and 143 g fat; the daily intake of pyridoxine was estimated at approximately 3 mg.† The spleen never became palpable. Admission blood studies showed a hypochromic anemia (4.5 g hemoglobin/100 ml) with normal leukocyte and platelet counts; the leukocyte differential count was within normal limits. Gastric analysis demonstrated 47 units free acid following histamine. A bone marrow biopsy was interpreted as showing some degree of erythrocyte maturation arrest but not consistent with that seen in classic Addisonian pernicious anemia. The anemia was unresponsive to parenteral liver injections (both purified and crude), pteroylglutamic acid, Brewer's yeast, iron and ascorbic acid in the dosages, routes and time duration of treatment indicated in Table I. Transfusions were given as necessary to maintain his hemoglobin at approximately 8 g/100 ml. Continuing studies (roentgenographic studies of the chest, upper gastrointestinal tract, small and large bowel, intravenous pyelograms; basal metabolic rate; stool for ova, parasites, blood excess fat or nitrogen; lymph node biopsy; blood chemistries, electrocardiogram) were done with normal or negative results.

In May, 1948 a spontaneous remission apparently occurred and repeated observations showed red count of over 4x10^6/mm^3 and hemoglobin of 12 to 13 g/100 ml. He was thereafter asymptomatic and received no therapy until November, 1953 at which time he again noticed onset of weakness and pallor.

Administration of cyanocobalamin and intravenous iron by private physician apparently did not alter progression of symptoms. In December, 1953 the hemoglobin was 8.5 g/100 ml; erythrocyte count 3x10^6/mm^3, hematocrit, 26%; reticulocyte count, 0.2% and the leukocyte count, 13.2x10^3/mm^3. The leukocyte differential showed 81% polys, 2% bands, 17% small lymphocytes; the platelet count was 420x10^3/mm^3; and blood urea nitrogen was 9.9 mg/100 ml. Tests for abnormalities found in various(11) hemolytic states were within normal limits including direct Coombs' test, electrophoresis of hemoglobin, alkaline denaturation for estimation of fetal hemoglobin, Donath-Landsteiner test, cold agglutination test, Ham test, bilirubin partition and total amount: no hemolysins or agglutinins could be demonstrated in the patient's serum in the pH range of 6.5 to 7.5. The indirect Coombs' test was positive at pH 7.5 but consistent with a transfusion subgroup incompatibility. The erythrocyte osmotic fragility was within normal limits; after 24 hours sterile incubation the osmotic fragility had shifted into the range indicating *increased resistance* to osmotic stress com-

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† We are indebted to Miss H. Ochi of the Dietetic Service of Crile Veterans' Administration Hospital for these calculations.

TABLE I. Agents Ineffective in Producing a Hematopoietic Response in Patient H.S.

Medication	Route admin.	Amt admin./day	Dates of treatment
Purified liver extract (15 U.S.P. units/ml)	I.M.	1 ml	1/28/47– 2/ 6
Crude liver extract (2 U.S.P. units/ml)	I.M.	2	4/21 – 4/30
	I.M.	5	1/ 8/54– 1/14
Bio-hepulin*	I.M.	2	5/14 – 5/28
Valentine's liquid extract of liver, U.S.P.	P.O.	30	4/21/47– 4/30
	P.O.	90	4/14/54– 4/28
	P.O.	120	4/29 – 5/10
Brewer's yeast tablets	P.O.	20 tablets	2/20/47– 4/30
Ferrous sulfate	P.O.	1 g	2/ 7 – 4/30
Cobaltous chloride	P.O.	180 mg	12/19 –12/24
Pteroylglutamic acid	P.O.	45	3/ 7 – 3/13
	P.O.	100	3/14 – 4/30
	I.M.	15	12/22/53–12/29
	P.O.	30	3/30/54– 4/12
Ascorbic acid	P.O.	150	3/ 6/47– 3/12
	P.O.	200	4/ 4 – 4/30
	P.O.	100	3/30/54– 4/12
Leukovorin	P.O.	30	1/ 2 – 1/ 8
	I.M.	6	12/30/53– 1/ 8/54
Cyanocobalamin	I.M.	100 μg	3/30/54– 4/12
	I.M.	1000	1/ 8/55– 1/30
Pantothenic acid	P.O.	10 mg	12/ 1/47
Cortisone	P.O.	100	1/15/54– 1/20
	P.O.	200	1/21 – 2/ 2
	P.O.	150	1/ 8/55– 1/28
Fresh human plasma	I.V.	1400 ml	6/ 8/54– 6/16
Vit. A	P.O.	75000 units	7/ 8/55– 8/28
D		3000	
Ascorbic acid		450 mg	
Thiamine		15	
Riboflavin		15	
Niacin		750	

* Extract prepared from pregnant mare's liver.

pletely outside the normal range. The erythrocyte mechanical fragilities determined before and after 24 hours of sterile incubation were only slightly increased above normal. An erythrocyte autosurvival time determined by Cr51 demonstrated a half life of 20 days; all the labelled cells had been removed from the circulation by 90 days. These values are significantly below the normal for the method (19). In addition to several agents which had been tried previously, the patient was treated with citrovorum factor, crude liver extract and 1400 ml of fresh plasma intravenously as indicated in Table I. To none of these therapeutic trials did he make any subjective, clinical or hematologic response. His hemoglobin level was maintained at approximately 7 g/100 ml by repeated transfusions of whole blood. In January, 1955 he was readmitted for a trial of cyanocobalamin and cortisone(12): there was no response to this combination of drugs. Transfusions were continued periodically until August, 1955 by which time he had received a total of 113 units of blood. He was then readmitted with an erythrocyte count of 2.86×10^6/mm^3, 5.9 g hemoglobin/100 ml, hematocrit 22%, leukocyte count 25.5×10^3/mm^3; the leukocyte differential showed the following percentage distribution: 76 polymorphonuclears, 16 small lymphocytes, 4 monocytes and 4 eosinophils. The mean corpuscular volume was 66 μ^3 and the mean corpuscular hemoglobin concentration, 27%. The erythrocytes on smear showed definitely abnormal variation in size and shape with frequent bizarre forms and

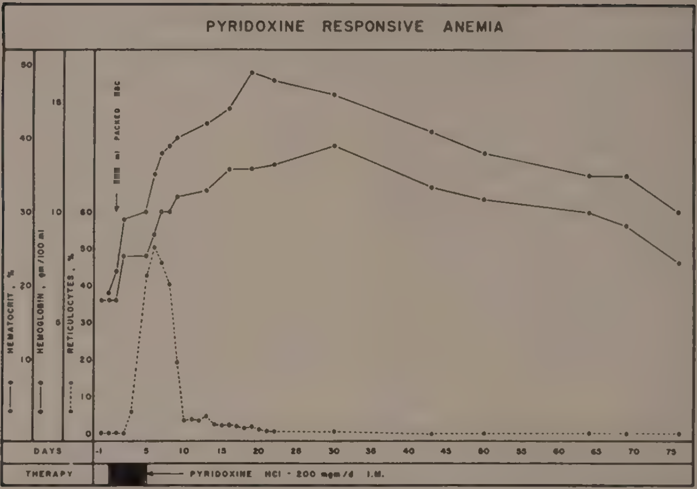

FIG. 1. Initial hematologic response of patient H.S. to intramuscular administration of pyridoxine and subsequent relapse following cessation of therapy.

target cells; the cells appeared hypochromic with decrease in hemoglobin content. During the previous 2 months the patient had been taking one tablet of a vitamin preparation 3 times a day. Each tablet contained: vit. A, 25,000 units; vit. C, 150 mg; vit. D, 1,000 units, thiamine chloride, 5 mg; riboflavin, 5 mg; and niacin, 250 mg. He was also taking quinine, grains 5 p.r.n. usually once every other week for night leg cramps; no other medicines were taken. A transfusion of 300 ml packed red cells was given on the same day pyridoxine hydrochloride, 200 mg intramuscularly daily was started. After 4 doses of this vitamin he noted a subjective improvement in general well-being and on the seventh day after institution of therapy, the reticulocytes had risen to 50.8%. Following this there was a rapid rise in the hematocrit and hemoglobin to maximum values of 49% and 13 g/100 ml respectively, on Sept. 12, 1955. The leukocyte count decreased to 9.2x10^3/mm. As soon as the reticulocytes were found to be elevated significantly, the pyridoxine administration was stopped, 5 doses having been given. By Nov. 14, 1955 the hematocrit had fallen to 27% and the hemoglobin to 7.8 g/100 ml. This response and subsequent relapse are outlined in Fig. 1. Before the response to pyridoxine the patient had complained of very troublesome nocturia, tibial pain and swelling of the feet constant throughout the day. Coincident with the reticulocyte rise the tibial pain disappeared and the edema subsided; shortly thereafter, the nocturia disappeared. After hematologic relapse when the hemoglobin had fallen to 7.9 g/100 ml and the hematocrit to 30%, the patient was started on pyridoxine, 1 mg intramuscularly daily. Except for the 1-tryptophan loading tests as indicated below, no medicines or transfusions were given at this time. No significant reticulocyte response occurred following the 1 mg pyridoxine dose and as indicated in Fig. 2, after 8 days it was increased to 10 mg intramuscularly daily. A reticulocyte peak of 17.7% on the seventh day of this therapy, was followed by a rapid rise in hemoglobin and hematocrit. Again, there had been subjective improvement coincident with the reticulocyte response. On Dec. 14, 1955 the hemoglobin reached 14.1 g/100 ml and the hematocrit 51.9%. The patient received 3 oral loading tests of 4 g 1-tryptophan, the first before pyridoxine therapy was started, the second during the 1 mg dose, and the final one shortly following the reticulocyte response during the 10 mg dose. Measurements of the urinary excretion of kynurenin, kynurenic acid, acetylkynurenin, xanthurenic

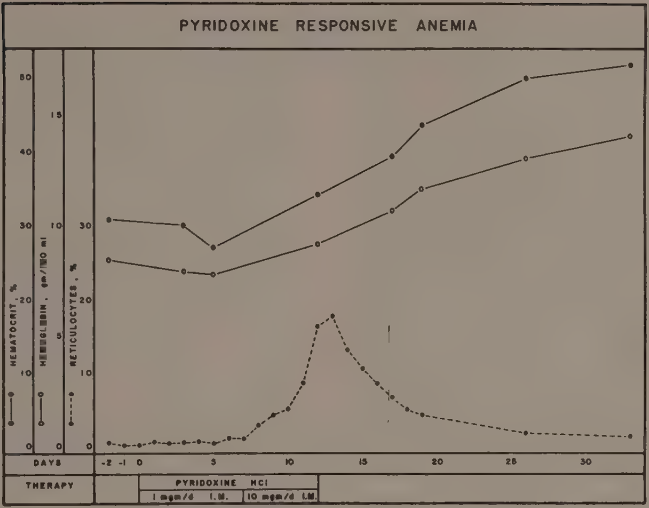

FIG. 2. Lack of hematologic response of patient H.S. to intramuscular administration of 1 mg pyridoxine daily with subsequent response to 10 mg pyridoxine daily. The l-tryptophan loading tests were done on days −2, 5 and 17.

acid, anthranilic acid glycuronide, ortho-aminohippuric acid, and n-methyl-pyridone-5-carboxymid indicated abnormalities of tryptophan metabolism(13). These abnormalities were partly reverted toward normal on the 1 mg pyridoxine dose and completely normalized at the 10 mg level(14). Before therapy the serum iron level was 170 µg/100 ml with a total iron binding capacity of 184 µg/100 ml and an iron binding protein saturation of 92%. Following therapy the serum iron was 67 µg/100 ml; the total iron binding capacity was 214 µg/100 ml and the saturation, 31%.‡

Discussion. Studies concerning pyridoxine have been accomplished in humans by eliminating the vitamin from the diet, counteracting the effect of the vitamin with an antagonist (4-desoxypyridoxine) and administering the vitamin as a therapeutic test(8). Although abnormalities in tryptophan metabolism have been readily produced(10), in only one instance has it been possible to show that pyridoxine deprivation in the human has resulted in altered hematopoiesis manifested by a severe microcytic hypochromic anemia, remediable by pyridoxine alone. Snyderman and associates(15) studied 2 hydrocephalic infants maintained on a synthetic diet devoid of pyridoxine; after 130 days anemia developed in one infant and abnormalities in tryptophan metabolism were demonstrated. These abnormalities disappeared after pyridoxine administration. No instances of naturally occurring abnormalities of hematopoiesis have been reported in humans that were responsive to the administration of pyridoxine. Hunt and associates(16) reported an infant who suffered from intractable convulsions which were controlled by pyridoxine. This infant was also described as having had a moderate anemia during the neonatal period; however, the only values reported for the first 23 days of life are: hemoglobin 13.1 g/100 ml and an erythrocyte count of 4.2×10^6/mm^3.

Employing the metabolic antagonist 4-desoxypyridoxine(17,18) dermatitis, glossitis, neuritis, lymphopenia and alterations in the handling of tryptophan and alanin were produced. However, the anemia that sometimes occurred could not be related to pyridoxine metabolism.

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‡ We are indebted to James A. Bowerfind, M.D., of University Hospitals, Cleveland, for these determinations.

Despite extensive empirical use of pyridoxine as a therapeutic test agent for many different conditions, its essential role in human hematopoiesis has been demonstrated only in the infant maintained on a synthetic diet lacking pyridoxine. The present observations indicate that abnormal hematopoiesis (associated with abnormalities in the metabolism of tryptophan and iron) can occur in the adult human secondary to a naturally occurring alteration in pyridoxine metabolism. Since the dietary intake of pyridoxine was estimated to be normal, the possibilities exist at present that either an absorptive defect or metabolic aberration accounts for the findings in this patient. The nature of the alteration and its role in the steps involved in hematopoiesis are unknown.

Summary. An adult patient is reported with hematologic abnormalities that were unresponsive to the usual hematopoietic agents, and characterized by a hypochromic anemia, leukocytosis, high serum iron and high per cent iron binding protein saturation. Measurements of the abnormal urinary excretion of the metabolites of l-tryptophan following oral loading tests demonstrated an alteration in the metabolism of this amino acid. The clinical and laboratory abnormalities were promptly reverted to normal, and hematological remission followed intramuscular administration of pyridoxine hydrochloride.

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Acceptance of Tumor Homografts by Mice Injected with Antiserum. II. Effect of Time of Injection.* (22285)

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With certain combinations of transplantable tumors and inbred strains of mice, a normally resistant host will accept a tumor homograft if the animal is injected with preparations of mouse tissues prior to tumor grafting(1-4). The altered response of the host may persist for many months after the

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tissue has been injected, but tissue administered *after* the tumor is grafted does not induce acceptance of the homograft(5). *Antiserums* to mouse tissues, produced in rabbits or mice, also will ensure survival of a tumor homograft when injected into normally resistant mice(6-9). The present report is a study of the survival of tumor homografts in mice receiving antiserum at different time intervals, with respect to the time of tumor grafting. It was undertaken to establish a comparison with the results previously found for the effects of varying the time of injection of *tissue* preparations(5). This comparison is of importance for an understanding of the mode of action of both the tissue injections and the antiserums. A preliminary report of the present findings has been presented elsewhere(10).

Materials and methods. The transplantable tumor used as the homograft was Sarcoma I (SaI) which is indigenous to the inbred A strain of mice. Hosts were mice of the C57BL/6Ks strain (a C57Black subline), which is genetically unrelated to the A strain. The tumor grows progressively in 100% of strain A mice, and very rarely in an untreated C57BL/6Ks mouse. (A full description of the properties of this tumor has been given previously(11).) All mice were 2 to 4 months old at the start of the experiments. *Antiserums* were produced in rabbits and C57BL/6Ks mice. The rabbits received injections of either freshly secured spleens of strain A mice or freeze-dried SaI tumor. The tissues were prepared as homogenates in sterile 0.85% saline, in an amount of approximately 20 mg/ml dry weight and the homogenates were centrifuged at 4500 x g. The supernatants thus obtained were used for intravenous injection, and the sediments for intraperitoneal injection. The rabbits received a total of 6 injections, spaced 4 days apart. Except for the first injection, in which supernatant was given intravenously, the sediment was injected in the morning, and the supernatant on the afternoon of the same day, to minimize anaphylaxis. The amounts of sediment injected per rabbit were, respectively, 1.0, 1.5, 2.0, 2.0, and 2.0 ml; all injections of supernatant were in the amount of 0.5 ml. The rabbits were bled from the heart while under nembutal anaesthesia. The serums were separated by centrifugation, freeze-dried and stored *in vacuo,* under refrigeration. For use in the experiments, the serums were reconstituted to the original volume with sterile distilled water. They were then heated at 56°C for 1 hour, since it had been found (12) that unheated rabbit antiserum may induce severe shock in mice, with subsequent death, and that the shock is greatly reduced by heating the serums. At the time that they were reconstituted, the serums had been in storage for 2 years and 8 months. In mice, centrifuged supernatants of freeze-dried or frozen SaI tumor were administered in 5 intraperitoneal injections of 0.5 ml each, over a period of 2½ weeks. Each mouse received a total of approximately 7.5 mg dry weight of tissue. Twelve days after the last injection, the mice were bled by incision of the lateral tail vein. An additional bleeding was made 1 month later, after a single "booster" injec-

TABLE I. Effect of Time of Injection of Rabbit Antiserums on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.

Interval between inj. of serums and t g*	No. mice dying with tumors; inj. with antiserums from rabbits No.†		
	K3	208	
Serum inj. before t g			
28 days	0/ 5	1/5	
7 "	0/ 5	4/5	
3 to 6 hr	10/10	5/5	
	Serum from rabbits No.		
	2P31	2P26/304‡	
3 to 6 hr before	5/ 6	13/13	
Serum inj. after t g			
1 day	2/ 6	3/ 5	
2		0/ 5	
3	0/ 5		
5		0/ 5	
7	0/10		
	Normal rabbit serum	Rabbit antiserum to guinea pig spleen	No inj.
4 hr before t g	0/6	0/6	0/27

* t g = tumor grafting.

† Numerators are No. dying with progressively growing tumors; denominators, total No. of animals in each group.

‡ Pooled serum from 2 rabbits.

tion of an equivalent of approximately 4 mg dry weight of tumor supernatant. All the serums were pooled and stored at −26°C. In some of the experiments, the isoantiserums had been produced shortly before they were used; in others they had been in storage for 10 months. The older serums were fully as effective as the fresher ones. Antiserums were administered intraperitoneally to the test animals. The homografts (SaI) were inoculated subcutaneously by trocar, under aseptic conditions, in the suprascapular region. Growth of the grafts was followed by periodic palpation until the mice either died with a progressively growing tumor, or remained without an evident growth for a consecutive period of 2 months, at which time the animals were classified as "negative."

Results. Serums produced in rabbits. (Table I). Four different lots of antiserums were tested, 3 of these from 3 different rabbits, and the fourth a pooled serum from 2 other rabbits. Rabbit K3 (Table I) had been immunized with the strain A tumor, SaI; the other rabbits were immunized with spleen from strain A mice. The serums from rabbits K3 and 208 were administered in 2 injections, per mouse, of 1 ml each, given 24 hours apart. The other 2 serums were administered in a single injection of 1 ml per mouse. The following points are evident from the data: (a) the best results, in terms of homograft survival, were obtained when the antiserum was injected shortly before tumor grafting; and (b) antiserum injected more than one day after tumor grafting did not effect homograft survival. There is a positive correlation between the relative strength of the antiserums from rabbits 208 and K3 and the length of time that their effectiveness remained in evidence when injected prior to tumor grafting. The serum from rabbit 208 exhibited both a higher complement-fixing titer and hemagglutinating titer (with red cells from strain A/Ks mice) than did the serum from rabbit K3. The data of Table I show that the serum from rabbit 208 exhibited some effect at an interval as long as 28 days between its injection and subsequent tumor grafting, while no homografts survived in the mice that received the K3 serum at as short an interval as 7 days before tumor inoculation. However, that there was some effect of the K3 serum given at this time interval was shown by the markedly larger transient growth of the grafts in this group of mice, and their longer persistence before regression, as compared with uninjected controls.

That the results described are due to specific antibody in the passively transferred antiserum is further shown by the lack of effect of normal rabbit serum, or rabbit antiserum to guinea pig spleen, and by data from a previous study on serum fractions(8).

Serums produced in C57BL/6Ks Mice (Table II). The isoantiserum was administered in a single injection of 1 ml per mouse. As with the antiserums produced in rabbits (Table I), the effect of the isoantiserums was more marked as the time interval of their injection, prior to tumor grafting, was decreased. The effectiveness of the isoantiserum, however, remained in evidence for a longer time interval than did the rabbit antiserum. With the isoantiserum, there was a marked transient growth of the grafts (as compared with untreated controls) in the mice injected as long as 9 weeks prior to tumor grafting. This was particularly evident in the male hosts, in whom palpable

TABLE II. Effect of Time of Injection of Isoantiserum, produced in C57BL/6Ks Mice, on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Mice.

Interval between inj. of serum and t g*	No. mice dying with tumors ♀	♂
Serum inj. before t g		
9 wk	0/ 5	0/ 5
5	0/ 5	3/ 5
1	5/ 5	5/ 5
4 to 6 hr†	14/15	20/21
Serum inj. after t g		
1 day	—	5/ 5
3	—	5/ 5
7	—	4/ 5
Normal C57BL/6Ks serum, 4 to 6 hr before†	0/ 5	0/17
Nothing inj.	0/ 5	0/10

* t g = tumor grafting.
† Data of 4 experiments.

grafts were still present 18 days after tumor inoculation, while in the untreated controls the grafts had completely regressed by 13 days.

In the group receiving isoantiserum 5 weeks before tumor grafting, there was a marked transient growth of the tumors in all 5 males. In 3 of the 5 males, the grafts resumed progressive growth after a slight regression, with eventual death of the mice. In the other 2 males, eventually classified as "negative," tumor nodules were palpable for 25 days after grafting. The grafts in the 5 females in this group also showed a large growth before regression, as compared with untreated controls, though they did not reach as large a size as in the males.

The difference between the sexes in the rate of growth of the grafts was also evident in the groups receiving isoantiserum 1 week before, or on the same day as, tumor inoculation. There consistently was a more rapid growth of the grafts in the males. Another difference between the effect of the isoantiserum and the rabbit antiserums was the survival of homografts in the mice receiving isoantiserum as long as 7 days *after* tumor inoculation. No homografts had survived in the mice receiving rabbit antiserum more than 1 day after tumor grafting.

Effect of dosage of antiserum and time of administration. The assumption was tested that dosage of antiserum might account for the differences observed between the effectiveness of rabbit antiserum and isoantiserum administered *after* tumor inoculation. This assumption was based on two premises: that more antibody is needed to insure the survival of a homograft after the immune responses of the host had been initiated; and that more antibody of the type ensuring homograft survival would be found, per unit volume, in isoantiserum than in the rabbit antiserum. Two groups of experiments were done, one with the gamma-globulin fraction of rabbit antiserum, and the other with whole isoantiserum produced in C57BL/6Ks mice. The gamma-globulin was used because it was known that it is active in inducing homograft survival (8). Furthermore, it was felt that the mice would tolerate better larger doses of the gamma-globulin than of whole serum, since it had been found that whole rabbit antiserum, even though heated to 56°C, will induce some degree of shock, and that the gamma-globulin fraction does not produce this reaction(12). Gamma-globulin was prepared by salt fractionation of an antiserum to spleen from strain A/Ks mice. It was then freeze-dried and reconstituted to the desired concentrations, on a dry weight basis, with sterile 0.85% NaCl.

TABLE III. Effect of Dosage and Time of Injection of Rabbit Antiserum Gamma-Globulin on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.*

Time γ-globulin inj. after t g†	No. mice dying with tumors at γ-globulin dosages/mouse of			
	10 mg (Exp. 1)	5 mg (Exp.1)	1 mg	
			Exp. 1	Exp. 2
Same day	—	—	5/5	5/5
1 day	5/5	5/5	5/5	—
3	—	—	—	0/5
4	3/4	2/5	1/5	—
5	—	—	—	0/5
7	2/5	0/5	1/5	—
Nothing inj. 3, 5, 7, 9 days after t g‡		1/5 (Exp. 1) 0/5 (Exp. 2)		

* Gamma-globulin obtained by fractionation with $(NH_4)_2SO_4$.

† t g = tumor grafting.

‡ 4 inj./mouse; 1 mg/inj.

The data for 2 separate experiments with gamma-globulin are given in Table III. In the groups receiving globulin at 4 or 7 days after tumor grafting, there is a positive correlation between the amount of globulin administered and the number of mice dying with progressively growing tumors. A marked growth of the grafts, which was initiated several days after the injection of globulin, was observed in almost all the mice in these same groups, whether or not the grafts were destined to grow progressively. In many of the grafts that eventually grew progressively, to death of the host, this spurt in growth was followed by a progressive diminution in size and then a resumption of growth.

The experiment with the isoantiserum produced in C57BL/6Ks mice was carried out at 3 different dosage levels of serum, the amount administered per mouse being given as a single injection of either 1.0 ml, 0.5 ml, or 0.25 ml. The data are presented in Table IV.

TABLE IV. Effect of Dosage and Time of Injection of Isoantiserum, Produced in C57BL/6Ks Mice, on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.

Time serum inj. after tumor grafting	No. mice dying with tumors, at serum dosages/mouse of 1 ml	0.5 ml	0.25 ml
2 days	5/5	5/5	5/5
4	4/5	3/5	2/5
7	3/5	3/5	1/5
10	1/5	3/5	2/5
14	0/5	0/5	0/5

There is no clear-cut evidence of differences due to dosage. In the groups of mice that died with tumors, and that had received antiserum 4 days, or longer, after tumor inoculation, there characteristically was an initial growth followed by a diminution in the size of the grafts, even for some time after the mice had received the antiserum, followed by a gradual resumption of growth. This experiment demonstrates, incidentally, that the grafts may remain viable in the foreign host for at least as long as 10 days after inoculation, a finding which is in agreement with previously published reports(5,13).

Discussion. The time relationships obtained with the antiserums are in contrast with those obtained in mice injected with tissue extracts(5). Homografts survived in mice inoculated with a tumor graft as long as 11 months after the mice had received an injection of tissue. On the other hand, tissue injected more than one day *after* tumor grafting did not induce homograft survival. With the *antiserums*, there was a diminution of their effectiveness as the interval of time between the administration of the serum and the subsequent grafting of the tumor was increased. This undoubtedly is attributable to the continuous elimination of the serum by the host. The injection of antiserum as long as 4, 7, or 10 days *after* tumor grafting ensured survival of a significant proportion of the homografts.

These observations, together with data obtained with cortisone-treated mice(9,14), indicate that the production of antiserum in mice pretreated with tissues may be a necessary condition for the survival of the tumor homografts. (Though the data show that the effects of the antiserums produced in rabbits and mice are the same, the possibility that there are qualitative differences in their mode of action is not ruled out.)

Three alternative hypotheses may be considered as to the mode of action of the antiserum. (a) The antiserum "coats" the specific tumor antigens which normally would evoke a "homograft reaction" by the host. Such coated antigens presumably would not stimulate the tissues (lymph nodes(13), and possibly other tissues) which otherwise would react to destroy the graft. (b) The antiserum acts directly on the reactive centers (lymph nodes, etc.) of the host to block the "homograft reaction," whether or not the animal had been previously exposed to the immunizing stimulus of a tumor inoculum. (c) The antiserum acts directly on the graft, inducing an adaptive alteration which permits the graft to survive in an otherwise hostile environment.

Our findings make hypothesis (a) untenable, since antiserum injected at 4, 7, or 10 days *after* tumor inoculation, led to survival of the grafts. That these time intervals should be sufficient to develop the immune responses of the host has been shown by Mitchison(13), who demonstrated that immunity to a tumor homograft could be passively transferred by lymph nodes taken from mice 3 to 10 days after the donors had been exposed to the immunizing stimulus of a homograft inoculum.

Whether hypothesis (b) is valid cannot be established from the data of the present paper. However, from data of other experiments it is believed that this hypothesis is open to question. A fuller consideration of this point has been given elsewhere(14).

The possibility that hypothesis (c) is reasonable is supported by the reports of Barrett and Deringer(15,16), and our own data (12), that a tumor homograft that survives in mice of a "foreign" strain loses some of its transplantation specificity, since it will grow progressively when grafted to untreated mice of inbred strains in which it normally would be rejected. Barrett and Deringer used a transplantable tumor, indigenous to the C3H strain, that had been grown in F_1 (C3H x C) mice. Though the tumor grew in 100% of the F_1 mice, these hosts can be considered as "foreign" to the tumor to the extent that half of the animals' heredity came from strain C parentage. When grafts derived from the "F_1" hosts were implanted into backcross (F_1 x C3H) mice, three times as many mice died with progressively growing tumors, as compared with backcross mice receiving their grafts directly from C3H mice. Barrett and Deringer(15) concluded that " . . . for the moment it is best to describe (the change in the "F_1" tumors) more loosely as an induced adaptation in the tumor." In our own case, the altered grafts came from a strain of mice in which survival of the tumors had been induced by pretreating the hosts with freeze-dried tumor tissue. On subsequent transplantation, the tumors grew progressively in untreated mice of an inbred strain which normally rejects the tumor. However, for reasons unknown, this change in graft specificity was lost after 7 transplant generations. An accelerated tumor growth in mice pretreated with killed tumor tissue, even in animals of the strain to which the tumor is indigenous, has been reported by Casey, Laster, and Ross (17). This characteristic was retained in subsequent transplant generations. Whether these examples are pertinent to our case would depend both upon the demonstration of the production of antiserum under the experimental conditions used by these investigators, and the effects of such an antiserum, if it is produced, upon the growth characteristics of the tumor grafts.

Another point brought out by our data is that the effects of the antiserums injected prior to tumor grafting are indicated not only by those cases where the grafts grew progressively, with eventual death of the host, but also by the numerous instances in which there was a marked transient growth of the grafts before they regressed. The latter cases are most probably an expression of subminimal doses of antiserum present in the host at the time of tumor inoculation. It may be assumed that the number of tumor cells affected by the antiserum was insufficient to ensure continued progressive growth of the grafts.

In conclusion, it is emphasized that a definitive answer as to the mode of action of the antiserum must await further experimentation, and that the mechanisms involved may be concerned with processes as yet unknown, and perhaps other than those that have been considered in this paper.

Summary. Passively transferred antiserum to mouse tissues, produced in rabbits or mice, will induce the survival of a tumor homograft in mice. The best effect, in terms of homograft survival, is obtained if the antiserum is administered to the prospective host shortly before, or shortly after, tumor grafting. The possible mode of action of the antiserum in ensuring homograft survival is considered.

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Failure of Zymosan to Increase Survival in X-Irradiated Mice.* (22286)

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Ross *et al.*(1) stated that the intravenous injection of zymosan in rats and mice was beneficial in radiation injury if given prior to irradiation, and detrimental if given afterwards. Although the effect obtained was related to the dose administered, the dosage range was not given. The mode of action of zymosan is related to its ability to combine with properdin, decreasing then increasing the serum content of this material. Properdin increases survival of irradiated animals by decreasing the post-irradiation bacteremia. Pillemer and Ross(2) gave information concerning the effects produced by doses of zymosan varying from 5 to 125 mg/kg. Finally Pillemer(3) reported that small intravenous doses of zymosan, administered to CF 1 mice, 24 hours pre- or post-irradiation, protected 70% of the animals against an $LD_{100}/_{30}$ day dose of x-irradiation. These investigators did not give the characteristics of the irradiation used or the manner in which the mice were restrained during irradiation. Regardless of the paucity of information, the report of any therapeutic agent which is beneficial in radiation injury requires that further experiments be undertaken by other laboratories. We have conducted such experiments using both low and high doses of zymosan and are unable to substantiate the beneficial effects reported by Ross and Pillemer.

Methods. Six-hundred male, CF 1 mice, weighing an average of 24 g each, were arranged in groups of 20 animals each according to the design given in Table I. The Fleischman yeast zymosan solutions were prepared according to Pillemer and Ross(2). Two series of animals received intravenous injections of 5 and 125 mg/kg respectively, while another series received a 5 mg/kg injection intraperitoneally. Except during irradiation the animals were maintained in an air-conditioned room at 72 ± 5°F and were fed a diet of Rockland pellets supplemented weekly with additional vit. A and D. The 550 r radiation dose was administered from above and below the mice with two 250 KVP Picker Industrial Units operating simultaneously. The technical factors were: 250 KVP; 15 ma; FOD 100 cm; filters, 0.21 mm Cu inherent, 0.5 mm Cu parabolic and 1.0 mm Al; HVL 2.02; size of field—total body; r/min measured in air 17.5 to 18.2. Both units were calibrated before and after each experiment with a Victoreen thimble r-meter. The animals were restrained in a plastic cage similar to the one described for guinea pigs (4). The results obtained were analyzed statistically by the Litchfield method(5).

Results. The results obtained with 5 mg/kg and 125 mg/kg of zymosan intravenously are given in Fig. 1 and Table I respectively. Intraperitoneal injection gave results similar to those in Fig. 1. The responses of the saline control groups in all series were similar to results obtained previously with other drugs(6-11). Post-irradiation medication with 5 mg/kg of zymosan either intraperitoneally or intravenously was highly detrimental to the mice, significantly reducing the ST_{50} day and day of total mortality in all cases. Pre-medication did not increase the ST_{50} day and the apparent 20% survival in the 7 day group is of doubtful significance because such effects have been observed in the control groups in past experiments. In fact, only 75% of the control animals of the intraperitoneal series died by the 26th post-irradiation day.

In the series given 125 mg/kg of zymosan, post-irradiation medication on days 1, 3 or 7 was highly detrimental decreasing the ST_{50}

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TABLE I. Effect of 125 mg/kg of Zymosan Intravenously on Survival Time in Irradiated Mice.

			Final mortality	
Medication†	ST_{50}* and range in days	Slope and range	%	Day
Saline control rad day	10.9 (9.45-12.55)	1.39 (1.26-1.54)	90	21
Z, 14 day pre-R	7.55 (6.56- 8.68)	1.39 (1.25-1.54)	95	14
Z, 7 ”	10.4 (9.17-11.8)	1.34 (1.22-1.46)	90	21
Z, 3 ”	9.65 (7.71-12.1)	1.60 (1.32-1.93)	75	13
Z, 1 hr ”	11.8 (5.31-26.21)	1.57 (1.33-1.86)	75	20
Z, ” post-R	12.2 (8.19-18.19)	2.31 (1.64-3.26)	65	20
Z, 1 day ”	7.35 (6.39- 8.59)	1.38 (1.25-1.53)	100	18
Z, 3 ”	5.55 (5.31- 5.85)	1.12 (1.08-1.16)	100	9
Z, 7 ”	9.3 (8.65-10.0)	1.18 (1.12-1.25)	100	12
Z, NR control	—	—	0	21

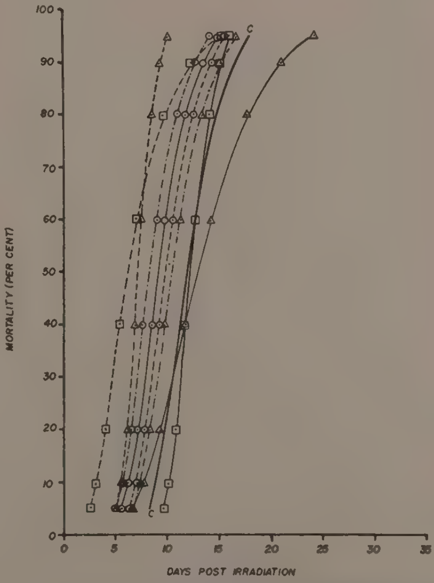
* ST_{50} = Day on which 50% of animals alive. All values at P, 0.05; 20 animals per group.

† Z = Zymosan; rad day = radiation day; pre-R = pre-irradiation; post-R = post-irradiation; NR = non-irradiated.

day as much as 5.5 days over the control value. Pre-irradiation medication on days 14 or 3 also significantly reduced the ST_{50} day. Injections given 7 days or one hour pre-irradiation or one hour post-irradiation produced no significant increase or decrease in the ST_{50} day. However, total survivals in the latter 2 groups were 25 and 35% respectively as contrasted to only 10% survival in the saline control group. The statistical significance of these total survival figures is doubtful because of previous work as well as the 25% survival in the saline controls in the intraperitoneal series. This is also borne out by the wide ranges of both the ST_{50} days and the slope values.''

Discussion. There can be little doubt that zymosan, in the doses and modes of administration reported herein, is not beneficial to irradiated mice. Smith(12) has also obtained similar results in mice. Thus, the work of Ross and Pillemer(1-3) cannot be confirmed. However, the conditions under which these investigators conducted their experiments have not been completely reported so that it is not possible to set up an exact duplicate of their work. On the other hand, the statistical design and overall range of conditions of the experiments reported herein should have been sufficient to uncover any beneficial effects which zymosan might have in radiation injury. Insofar as the mechanism of action of zymosan on the properdin system is concerned, the present work actually gives no insight into the problem because properdin titers were not measured. The increased rate of mortality observed with post-irradiation medication might have been the result of depletion of properdin in the blood. However, previous work from this laboratory(6-11) indicates that the irradiated animal reacts adversely to medication during the first post-irradiation week. Compounds like vit. E which are non-toxic in normal animals cause an increase in both the rate of and total mortality, if given after irradiation(7).

Summary. Pre-medication with 5 mg/kg of zymosan intravenously or intraperitoneally did not significantly increase survival in ir-



EFFECT OF ZYMOSAN ON MORTALITY OF IRRADIATED MICE.
TREATMENT: SALINE CONTROL ——, 5 MG/KG. I.V. ZYMOSAN 14 DAY PRE ⊙—⊙, 7 DAY PRE △—△, 3 DAY PRE ⊡—⊡, 1 HOUR PRE ⊙- - -⊙, 1 HOUR POST △- - -△, 1 DAY POST ⊡- - -⊡, 3 DAY POST ⊙-·-⊙, 7 DAY POST IRRADIATION △-·-△

FIG. 1.

radiated mice. With intravenous injections of 125 mg/kg on pre-irradiation days 14 and 3, a significant reduction in the ST_{50} day was observed. Similar results were obtained with both doses when injections were given after irradiation. The beneficial effects of zymosan in the treatment of radiation injury in mice reported by Ross and Pillemer could not be confirmed.

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Effect of Hyaluronidase on the Cat's Brain. (22287)

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The existence of interstitial spaces between elements constituting the nervous system has been assumed. Investigations of Bairati and Mattioli(1,2) confirmed and proved the existence of these interstitial spaces which probably were designed for the flow of cerebrospinal fluid. The experiments of Bairati(3), Bairati and Tripoli(4), Bairati and Patterno (5), Bartoli and Bertaccini(6), and Massari and Marsico(7) demonstrated by histochemical tests on different groups of animals the existence of a ground substance, the main element of which is a mucopolysaccharide or a mucoprotein contained in the interstitial spaces of the nervous tissue. The results of Hotchkiss' reaction were clearly positive in nervous tissue of fishes, amphibia, and lower mammals, whereas the results were doubtful when the test was performed on the brain of birds and higher mammals. These authors studied the behaviour of the nervous tissue under the influence of hyaluronidase and proved that the ground substance is affected by hyaluronidase, which allows a more rapid and extensive diffusion of the injected liquids and an easier mechanical dissociation of fragments of nervous tissue. Thus mucopolysaccharide may be present in interstitial spaces of the nervous substance. Freedman(8) also proved by means of histochemical reactions the existence of a substrate hydrolyzed by hyaluronidase, probably hyaluronic acid, in the nervous system, *i.e.* in the cerebral cortex, nucleus caudatus, and thalamus. Recently, Hess(9) stated too that the ground substance of the nervous tissue is a mucopolysaccharide, rather than a mucoprotein; since it is not hydrolyzed by pepsin, trypsin, or pancreatin. Hess found that hyaluronidase had no effect, and this indicates that this substance is not hyaluronic acid. In the present work, the behaviour of the nervous tissue under action of the hyaluronidase enzyme was examined.

Materials and methods. 13 young adult cats of both sexes were used, with a weight of 1.5-2 kg. All animals were operated on under aseptic conditions and ether anesthesia. Symmetrical points of the skull were opened, and after incising the duramater, the following procedures were carried out. In the *first test,* on 2 animals, a superficial wound of the cerebral cortex was made by application of the trephine on both sides of the chosen area for 10 seconds. After stopping the hemorrhage, a square piece of paper 7 mm side length, soaked in physiological saline solution was applied to the control side. On the other side, another piece of paper of the same form and size was applied, which immediately before had been soaked in a recently prepared hyaluronidase solution (30 units of Schering's "Kinaden" in 0.1 ml of physiological serum). Both wounds were covered with a piece of cellophane paper applied to the bone between it and the muscles. After closing the wound, 10 ml of a solution of Merck's trypan blue of 1% in physiological serum was injected intravenously and repeated every 3 hours. The animals were sacrificed 10 hours later by bleeding under anesthesia. In the *second test,* 2 cats were treated in the same way, but the quantity of hyaluronidase used was 10 units. Trypan blue was 20 ml divided into 2 injections separated by 40 minutes. The animals of this group were sacrificed 10 hours later. In the *third test,* cerebral lesions were made by application to the cortex of a square piece of paper, 5 mm side length, soaked with carbol-xylol. This was kept in contact with the cortex for 40 seconds. Thereafter, the symmetrical points of the cortex treated in this way were irrigated with serum at 37° and then the animals were treated exactly as in the first test. One of the animals was sacrificed 2 hours after, and the remaining 2 after a period of 10 hours. In the *fourth test,* 4 cats were treated with hyaluronidase inactivated by heat, the enzyme having been dissolved in serum at 70° and the solution incubated one hour at 70°. The experimental procedure was the same as in the third test. Two animals of this group were sacrificed and the remaining ones 10 hours after. In the *fifth test,* in 2 cats a deep injection of 0.2 ml of trypan blue solution at 1% in physiological serum containing 20 units of Kinaden was injected deeply into the cortex on one side. In a symmetrical point of the cortex of the other side, a 0.2 ml of trypan blue dissolved in physiological serum at 1% was injected, to the same depth and the animals sacrificed after a period of 24 hours. The brains were fixed in an aqueous formol solution at 10% and afterwards examined microscopically with a magnifying glass.

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Results. In cats of the first test, a larger diffusion of the dye was seen on the side which had been treated with hyaluronidase (Fig. 1-A). The stain is broader on the treated side than on the control side to which physiological serum had been applied.

In the second test the results were analogous. On the side treated with hyaluronidase the dye extended over a broader area than that which was reached on the control side. The diffusion area was smaller in this test than in the first one.

In the third test, the results were exactly the same as in the previous ones.

In the fourth test, in which the hyaluronidase had been inactivated by heat, the diffusion area of the dye was essentially the same on both sides. (Fig. 1-B).

In the fifth test, the dye formed on the control side a small sediment of quite precise limits while on the side in which hyaluronidase had been injected, it had spread into the adjoining area, in the immediate nervous tissue following the needle's course and forming a halo of pale blue color.

Discussion. The behavior of the nervous tissue in the presence of the hyaluronidase enzyme was also studied by Bairati(10) using the method of injection—at low pressure—of coloring substances (India ink) diluted in solutions of the enzyme with physiological serum. Injections were performed in nervous tissue obtained immediately after death and in living animals. In the technic applied by Bairati, the quantity injected is not exactly determined and the pressure at which the injection is performed cannot be regulated with accuracy, a fact which possibly may influence the degree of diffusion of the coloring substance thus rendering doubtful the interpretation of the results.

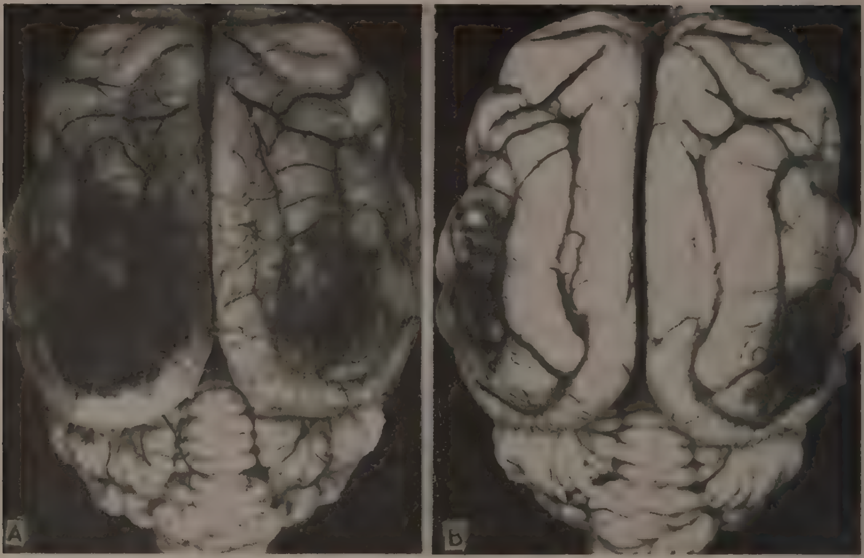

FIG. 1. A. First test. Left side treated with hyaluronidase. Right side (control) treated with physiological serum. Notice broader diffusion of coloring substance on left side. B. Fourth test. Left side treated with hyaluronidase inactivated by heat. Diffusion area of coloring element is approximately the same as on the right (control) side.

The technic used in our experiments avoids any such errors. Passage of the dye takes place through the blood vessels at the same pressure and at the same concentration on both sides, and the animal serves at the same time for test and control.

It can be deduced from our results that in the area of the cortex to which the enzyme was applied, the diffusion of the dyestuff is greater than on the control side. The degree of diffusion was influenced by concentration of the enzyme in the tested solution, and by the time over which it was allowed to act. This effect of the enzyme disappears when it is inactivated.

These results indicate the existence of a substance—probably hyaluronic acid—in the cerebral cortex of the cat which is susceptible to the action of the hyaluronidase.

Summary. 1) The existence of a substance susceptible to the action of hyaluronidase in the cat's brain is demonstrated. 2) The action of hyaluronidase on the cerebral substance is influenced by the concentration of the enzyme and by the duration of its action. 3) When the enzyme is activated by heat, the effects of the diffusion factor on the nervous system disappear.

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In vitro Metabolism of DL-Alanine-2-C-14 and Glycine-2-C-14 by *Trichinella spiralis* Larvae.* (22288)

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Recent work from this laboratory(1) has shown *in vivo* incorporation of carbon-14 by immature and encysted *Trichinella spiralis* larvae from mice fed C^{14}-labeled amino acids. When glycine-2-C-14 and dl-alanine-2-C-14-labeled diets were fed to mice with Trichinella infections of 14 and 56 days' duration, the larvae incorporated C^{14} indicating that they were exchanging metabolites with the host. Encysted larvae in 180-day infected mice also incorporated significant levels of C^{14} from the tissues of mice fed glycine-1-C-14 and dl-alanine-1-C-14 diets, showing an active metabolism by well encapsulated muscle larvae.

In the present study, Trichinella larvae were isolated from host tissues by pepsin digest and cultured in media containing dl-alanine-2-C-14 or glycine-2C-14. This report concerns *in vitro* incorporation of C^{14} by muscle larvae and metabolism of C^{14} into larval protein from C^{14}-labeled amino acids.

Materials and methods. Trichinella larvae were obtained from stock mice with infections of 4 months' duration. The muscle larvae were freed from their cysts by pepsin digest, separated from undigested materials and washed to remove soluble digestion products, as previously described(1). The Krebs-Ringer bicarbonate solution was prepared using the procedure outlined by Cohen(2). Normal mouse serum and Krebs-Ringer solution were used to prepare 4 types of media as follows: a) a 50-50 mixture of mouse serum and Krebs-Ringer solution containing glycine-2-C-14; b) a 50-50 mouse serum and Krebs-Ringer solution containing dl-alanine-2-C-14; c) Krebs-Ringer solution with glycine-2-C-14; and d) Krebs-Ringer solution with dl-alanine-2-C-14. A 2 ml sample from each medium was saved to determine C^{14} content prior to incubation with Trichinella larvae. Beckman pH meter determinations of the media gave pH values of 7.68 for the serum Krebs-Ringer media and pH 7.61 for the Krebs-Ringer media. The values for total C^{14} activity and free amino acid concentration in the media are shown in Tables I and II. Aseptic technic was used to prepare 4 series of 50 ml culture flasks containing 10 ml of the C^{14}-labeled media. Approximately 100,000 larvae were introduced into each culture flask by pipette suspension in 0.5 ml of 0.85% saline. Penicillin G Potassium (Lederle) 1,000 units and 1,000 μg of Dihydrostreptomycin Sulfate (Lilly) in 0.2 ml volume were added to each flask to control bacterial growth. Thus, each flask contained a total volume of 10.7 ml of the C^{14}-labeled media. The serum-Krebs-Ringer culture flasks were incubated at 37.5°C for periods of 3, 6, 12, 24, and 48 hours. The Krebs-Ringer culture flasks were incubated at the same time for periods of 6, 24, and 48 hours. During incubation the flasks were shaken at 50 cycles per minute. At the end of each incubation period, the larvae were separated from the culture media by centrifugation at 1,000 rpm. The larvae were washed 6 times in 50 ml distilled water and the supernatant drawn off by aspiration. Live Trichinella larvae settle out rapidly, while the dead larvae, if present, float and are lost in the supernatant. Microscopic examination of the washed larvae just prior to lyophilization revealed only motile larvae. The following procedure was devised to obtain total soluble and insoluble larval protein for C^{14} analysis. Lyophilized larvae (7 mg) samples were weighed in specially modified 12 ml centrifuge tubes. The tubes were fitted with removable base tips. The larval material in the base tip was suspended in 0.15 ml of 10% sodium tungstate

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TABLE I. Influence of Incubation Time upon Glycine-2-C-14 Metabolism by Trichinella Larvae.

Composition of glycine 2-C-14 media	Serum and Krebs-Ringer solution					Krebs-Ringer solution		
Total activity/flask (cpm)	3.808×10^6					3.1576×10^6		
Total wt of free glycine/flask (μg)	(86.7† + 95‡) = 181.7					71.9†		
Hr of incubation	3	6	12	24	48	6	24	48
Total wt in mg lyophilized larvae	40.14	34.11	37.22	31.24	26.09	35.01	36.22	27.80
Total wt in mg larval carbon	15.99	13.92	14.42	12.80	10.71	15.44	14.21	11.16
cpm/mg carbon lyophilized larvae	1,938	3,284	8,145	27,895	47,846	1,481	30,697	58,158
Total activity (cpm) lyophilized larvae*	30,998	45,713	117,451	357,056	512,430	22,867	436,204	649,043

* **These values represent total C^{14} content in larvae recovered from each culture flask.**

† Added glycine is calculated from No. of counts per flask and specific activity of glycine-2-C-14 (19.965 μc/mg).

‡ **Estimated from content of free glycine of normal mouse plasma(5).**

TABLE II. Effect of Incubation Time upon Dl-Alanine-2-C-14 Metabolism by Trichinella Larvae.

Composition of dl-alanine-2-C-14 media	Serum and Krebs-Ringer solution					Krebs-Ringer solution		
Total activity/flask (cpm)	1.143×10^6					0.776×10^6		
Total wt of free alanine/flask (μg)	(127† + 285‡) = 412					87†		
Hr of incubation	3	6	12	24	48	6	24	48
Total wt in mg lyophilized larvae	36.26	36.07	36.78	29.74	40.46	33.17	27.89	27.41
Total wt in mg larval carbon	15.85	15.38	15.85	12.77	16.92	14.79	12.26	11.42
cpm/mg carbon lyophilized larvae	190	525	734	901	1,602	214	2,055	2,300
Total activity (cpm) lyophilized larvae*	3,011	8,075	11,633	11,505	27,106	3,165	25,194	26,266

* These values represent total C^{14} content in larvae recovered from each culture flask.

† Added dl-alanine is calculated from No. of counts per flask and specific activity of dl-alanine-2-C-14 (4.08 μc/mg).

‡ **Estimated from content of free l-alanine of normal mouse plasma(5).**

and precipitated with 1 ml of ⅔ N H_2SO_4. The precipitated larval protein was centrifuged into the tip of the tube at 2,200 rpm. The material in the tip was resuspended in 0.15 ml sodium tungstate, precipitated again with acid and centrifuged. The process was repeated once more and the samples dried in a high vacuum oven at 30°C. The centrifuge tips containing the larval protein† were removed and placed in Van Slyke carbon combustion tubes for analysis. All lyophilized larvae and larval protein samples were analyzed for C^{14} using the methods of Van Slyke *et al.*(3,4).

Results. The first experiment was designed to test *in vitro* the ability of *T. spiralis* larvae to metabolize glycine-2-C-14 when cultured in a serum-Krebs-Ringer medium and a chemically defined Krebs-Ringer medium. The total activity (cpm) of C^{14} per flask and period of incubation for each culture are shown in Table I. It is evident from the data that the larvae actively incorporate glycine-2-C-14 throughout the 48-hour period of incubation in both types of media. The larvae cultured in the Krebs-Ringer medium incorporated more C^{14} than the larvae cultured in the serum-Krebs-Ringer medium. This is of interest in that the total activity (cpm) per flask was higher in the serum-Krebs-Ringer medium than in the Krebs-Ringer medium. This may indicate an acceleration of glycine-2-C-14 metabolism when it is the only amino acid present in the medium and also suggests that the larvae utilized unknown metabolites present in the serum. The amounts of serum free amino acid (glycine) and C^{14}-labeled glycine per flask are shown in Table I. When isotope dilution of the C^{14}-labeled amino acid by the free amino acids of serum is considered, the actual uptake of glycine by the larvae in the serum media may be greater than indicated by the C^{14} values. The incorporation of C^{14} by larvae in the Krebs-Ringer culture during the first 6 hours was less than half the amount found in the larvae incubated for 6 hours in the serum-Krebs-Ringer medium. Apparently, the larvae in the chemically defined Krebs-Ringer medium required a period of adaptation to the medium and in the following 6- to 24-hour period of incubation incorporated glycine-2-C-14 at a more rapid rate.

In the second *in vitro* experiment, the metabolism of dl-alanine-2-C-14 by Trichinella larvae was studied. The total activity (cpm) of C^{14} per flask and period of incubation for each culture are shown in Table II. The larvae were taken from the same pool of isolated muscle larvae used in the first experiment. The cultures were incubated at the same time as the cultures in the first experiment. The data in Table II indicate that the larvae were able to metabolize dl-alanine-2-C-14 although not as extensively as found with glycine-2-C-14. During the first 6 hours of incubation, the larvae incorporated more C^{14} from the dl-alanine-2-C-14 serum-Krebs-Ringer medium than from the Krebs-Ringer medium. Although the C^{14} activity (cpm) of the serum-Krebs-Ringer medium was higher than the Krebs-Ringer medium, the 6- and 24-hour period of incubation demonstrated a more rapid rate of C^{14} uptake by the larvae in the Krebs-Ringer culture, than in the serum-Krebs-Ringer culture. If, in the 48-hour serum cultures one assumes that the same proportion of the l-alanine, added with the free amino acids of the serum, is incorporated as of the l-alanine in the added C-14-dl-alanine, the total amount of alanine incorporated is estimated to be about 5 times the amount incorporated in the 48-hour Krebs-Ringer cultures.

Table III shows the per cent incorporation of C^{14} into total larval protein by Trichinella larvae cultured 48 hours in the 4 types of media. A higher concentration of C^{14} was found in the total protein obtained from larvae cultured in either glycine-2-C-14 or alanine-2-C-14-labeled Krebs-Ringer media as compared with the C^{14}-labeled serum-Krebs-Ringer media. Although the total C^{14} level of the serum-Krebs-Ringer media was higher than the Krebs-Ringer media (Tables I and III), the larvae incorporated less C^{14} into protein when they were incubated in the

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† The material precipitated by tungstic acid will be referred to as "protein"; it presumably includes lipids, purines and primidines of nucleic acids.

TABLE III. Influence of Composition of Media upon Protein Metabolism of 48 Hour Incubated Trichinella Larvae.

Amino acid	Glycine-2-C-14		Dl-alanine-2-C-14	
Media	Serum and Krebs-Ringer solution	Krebs-Ringer solution	Serum and Krebs-Ringer solution	Krebs-Ringer solution
cpm/mg of lyophilized larvae	19,634	23,362	670	959
cpm in protein from 1 mg lyophilized larvae	13,660	19,712	212	545
% C^{14} incorporated into larval protein	69.6	84.4	31.7	56.8

serum media, indicating that they utilized metabolites present in normal mouse serum.

The C^{14} activity from glycine-2-C-14 was incorporated into larval protein material in higher quantity than alanine-2-C-14 regardless of the media used. Glycine, in addition to its incorporation into protein peptides as glycine, may be converted into other amino acids which are eventually incorporated into protein. Furthermore, there is the possibility of incorporation of glycine into purines and pyrimidines of the nucleo-proteins. The higher values of C^{14} activity from alanine-2-C-14 in nonprotein larval material suggests that the larvae are able to convert alanine into nonprotein components, such a glycogen, glucose-1-PO_4 and others *via* the pyruvate pathway.

Summary. 1. *Trichinella spiralis* larvae incorporated C^{14} when cultured *in vitro* in a serum-Krebs-Ringer medium or Krebs-Ringer medium containing either dl-alanine-2-C-14 or glycine-2-C-14. 2. Carbon-14 analysis of the larvae revealed a progressive uptake of the C^{14}-labeled amino acids in both types of media through 48 hours of incubation. 3. The larvae incorporated more C^{14} activity (cpm) from glycine-2-C-14-labeled media than from dl-alanine-2-C-14-labeled media. 4. Of the total C^{14} incorporated by larvae cultured 48 hours in glycine-2-C-14-labeled media about 70 to 84% was precipitable by tungstic acid, and was presumably chiefly in the proteins. Larvae cultured in the dl-alanine-2-C-14-labeled media incorporated about 32-57% of their total C^{14} content into material precipitable by tungstic acid.

We wish to acknowledge the technical assistance of Rhoda Palter and Alina Jakubowski.

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Effect of Intravenous Administration of Lactate on Blood Pyruvate Level in Man. (22289)

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The role of circulating lactate in body metabolism is not entirely clear. On the one hand, the concept of the Cori cycle implies that most or all circulating lactate becomes glycogen in the liver; on the other hand, there is ample evidence that lactate can serve as a source of energy in some organs(1). Knowledge of whether the blood pyruvate level changes after intravenous infusion of lactate should afford some evidence concerning whether or not lactate enters the general metabolic pool. Accordingly, the former was studied in normal human subjects.

Material and methods. Eight subjects, ranging in age from 18 to 30 years, were used; 3 were women. All were well nourished and had taken, in addition to their usual diets, added amounts of carbohydrate for at least three days before receiving the lactate infusion. The studies were made with subjects recumbent after a fast of 14 hours and a rest of at least half an hour. Each subject was given an intravenous infusion of 17.4 g of sodium d-lactate, dissolved in 500 ml of isotonic sodium chloride solution, in 30 minutes. Blood samples were taken before, and at 15-minute intervals for one hour after, the start of infusion. The method of Barker and Summerson(2) was used to measure blood lactic-acid concentration; the method of Seligson and Shapiro(3) was used to measure blood pyruvic and α-ketoglutaric acid concentrations. In addition, 4 subjects, including one who had received a lactate infusion, were given 50 g of glucose intravenously under similar experimental conditions; blood pyruvate concentrations were measured before and immediately after infusion.

Observations. The blood pyruvate level rose during and after infusion of sodium d-lactate (Fig. 1); the average rise at the end of infusion was 0.55 mg per 100 ml of blood, or 89% of average control value of 0.62 mg per 100 ml. After infusion blood pyruvate level usually fell more slowly than did blood lactate concentration (Fig. 1).

Blood α-ketoglutaric-acid concentration rose in 6 experiments (including 2 in which a slight initial fall occurred). It was unchanged in 1 and fell in 1 during and after lactate infusion (Fig. 2).

Intravenous administration of 50 g of glucose in 30 minutes caused no rise in blood pyruvate level in any of four experiments.

Discussion. Increasing circulating blood lactate concentration in normal man caused a striking increase in blood pyruvate level. There was no good correlation between rise

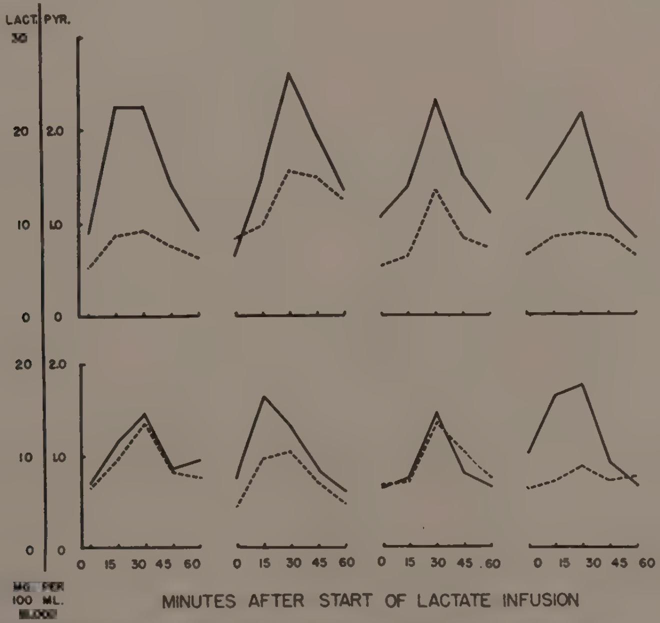

FIG. 1. Changes in blood lactic (solid line) and pyruvic (broken line) acid concentrations during and after infusion of sodium d-lactate.

in blood lactic acid level and that of blood pyruvic acid concentration. Of 3 subjects who showed smallest increases in blood pyruvate concentration, 1 excreted large amounts of lactate in the urine. The other 2, on the other hand, showed large increases in blood lactate; hence urinary excretion of that substance could not account for the small rises in blood pyruvate concentration.

The present data allow no conclusions about quantitative relations between lactate administration and pyruvate formation, for several reasons: 1) Varying amounts of lactate were excreted in the urine; 2) there is no evidence that rate of removal of pyruvate from circulation was the same in all cases; and 3) it is probable that rate of transformation of lactate to pyruvate was not uniform among the subjects. No information is available concerning the site where lactate was changed to pyruvate under conditions of the experiment.

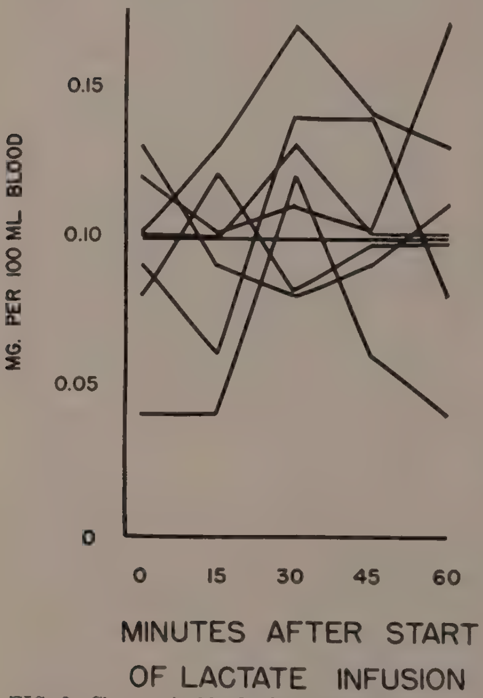

FIG. 2. Changes in blood α-ketoglutaric acid concentration during and after infusion of sodium d-lactate.

It is evident that circulating lactate can contribute rapidly and in significant degree to the general metabolic pool in resting, fasting, well-nourished man. The rise in blood α-ketoglutaric-acid level that usually accompanied the rise in pyruvic-acid concentration supports this conclusion. Actually, the rise in blood pyruvate concentration that occurred after a half-hour infusion of 17.4 g of lactate was far greater than that which occurred after a half-hour infusion of 50 g of glucose. This fact suggests that lactate enters the metabolic pool far more rapidly than does glucose.

Conclusion. The striking rise in pyruvate concentration that accompanies the infusion of lactate in fasting, resting human subjects indicates that circulating lactate contributes to the general metabolic pool.

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Cytological Response in Embryonated Eggs to Inoculums from Cases of Common Cold Infection.* (22290)

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Studies on common cold infections have involved principally transmission studies in human volunteers. Frequently, the results of such studies have been difficult to evaluate because of the variable human susceptibility and the indecisive clinical criteria for diagnosis of the common cold syndrome. Human inoculation experiments have served to demonstrate that common cold infection is a transient air borne viral disease(1,2), limited in pathogenicity to man and ape,(3,4). Propagation of the etiological agent in cultures of human tissues has been reported(5). Propagation of common cold agents in embryonated eggs has been affirmed(6-10) and denied (4,11) but in all instances the decisions rested on the response of human volunteers to experimental inoculations.

Recent observations have indicated that the propagation of influenza and mumps viruses in embryonated eggs was accompanied by an increase in numbers of histiocytes in the allantoic fluid (AF). Parallel studies by hemagglutination and histiocyte counts indicated that both of these effects occurred simultaneously. This non-specific cellular reaction was inhibited by antiserums mixed with the viral agents prior to inoculation, thereby affording some basis for identification of the agent. Using this as a reference point, less well recognized pathogens were studied for similar effect in AF. By following the cytological response in serially passaged AF in inoculated eggs, this effect was demonstrated with serum inoculums from acute cases of clinically diagnosed infectious and serum hepatitis(12,13). Nasal washings from clinically diagnosed cases of common cold were similarly studied in embryonated eggs and increased numbers of histiocytes in the AF were associated with such inoculums(14). Control inoculums from cases of allergic rhinitis have been negative. The experimental data on which these observations were based are described below.

Methods. Standard strains of influenza A, A^1, and B viruses† were each inoculated into the AF of six 9 day embryonated eggs. After 2 days of additional incubation the eggs were stored at 4°C overnight and blood-free AF was aspirated by syringe and needle. A 20 ml aliquot of each pooled AF was centrifuged at 2000 rpm for 20 minutes, the supernatant fluid was quickly decanted, and the sediment was resuspended in the residual fluid. A standard "Breed" loopful of the sediment was transferred to a 1 sq cm area on a clean glass slide. Duplicate smears of each specimen were air dried, stained with Wright's stain, and air dried. The total number of histiocytes in 100 consecutive fields was determined with a 5 X ocular and an oil immersion lens. The histiocytes referred to are mononuclear basophilic cells in which a distinct cytoplasm can be detected. In contrast to the rigid cell wall of the erythrocyte line of cell, the histiocyte was undulant. Blood in the AF was minimized by killing the embryos with refrigeration. Groups of 7 day embryonated eggs were inoculated into the chorioallantoic cavity with allantoic fluid containing mumps virus (Enders strain). Four days later the inoculated eggs were stored overnight in the refrigerator and the AF, collected on the morning, was processed for histiocyte content and hemagglutination titer. Controls for such inoculations consisted of normal AF, fluids from eggs previously inoculated with normal AF, and heated influenza and mumps viruses (61°C/30 minutes). The AF specimens were simultane-

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* Supported by funds provided under contract with the USAF School of Aviation Medicine, Randolph Field, Texas.

† Obtained from American Type Culture Collection, Washington, D.C.

ously examined for hemagglutinins with chicken erythrocytes(15). Antiserums from chickens, specifically immunized against the 3 influenza types, were mixed equal parts with viral inoculums and their *in ovo* effects on hemagglutinin production and histiocyte counts were determined. Nasal washings were collected from 21 human patients (medical students) having acute afebrile rhinitis, a syndrome coinciding with the conventional description and history of common cold. These cases occurred during the winter season and by careful selection those having a history of allergic rhinitis were excluded. Nasal washings were collected within 12 hours after onset of illness and a second specimen was collected 2 weeks later when the patient was asymptomatic. Five ml of sterile rabbit serum (2%) in physiological saline was instilled into the nostrils of the reclining patient and the fluid was permitted to run from the nostrils into a petri dish when the patient sat up. The nasal washings were treated with penicillin (1200 units per ml) and streptomycin (100 μg per ml), then 0.1 ml was inoculated into the allantoic cavity of six 7-day embryonated eggs. The shell holes were sealed and the eggs were incubated for 4 additional days at 38°C. The live embryos were then killed by overnight storage at 4°C. The allantoic fluid was collected and examined for histiocytes by the technic described above and the number of histiocytes per 100 oil immersion fields was recorded. Serial passages of AF were made with 0.1 ml into the allantoic cavity of 7-day eggs. Control inoculations consisted of normal allantoic fluid, heated inoculums (61°C/30 minutes), and convalescent nasal washings. Additional control inoculums consisted of nasal washings from 23 individuals with acute allergic rhinitis. The diagnosis was based on a history of allergic episodes following exposure to known allergens and on the microscopic detection of numerous eosinophilic leukocytes[‡] in the nasal effluent. The nasal washings were collected in physiological saline and stored at −20°C and subsequent manipulation in eggs followed the technic described above.

It is essential to the accuracy of the test procedure that eggs of superior quality be incubated under carefully controlled conditions, especially with regard to temperature and humidity. Abnormal number of histiocytes were encountered in the AF of uninoculated eggs when they were exposed to high temperatures during the shipment to the laboratory. The eggs used during the cool time of the year were uniformly satisfactory. Experimental procedures were performed at least in duplicate and all fluids were cultured for bacterial content in thioglycollate medium.

Results. The propagation of influenza and of mumps viruses was accompanied by the appearance of hemagglutinins and of histiocytes in the AF (Tables I and II). The cell counts significantly exceeded the normal levels (Fig. 1). Both histiocyte and hemagglutinin production were specifically reduced by treatment of inoculums with homologous antiserums (Table I). Histiocyte counts and hemagglutinins were negative at the same titration end-point in eggs (Table II).

Antibiotic-treated nasal washings from 15 of 21 common cold patients induced increased numbers of histiocytes in the AF of inoculated eggs. Hemagglutinins could not be detected. The embryos did not die as a result of such inoculations. The counts ranged from borderline with 5 specimens to marked increases with 10 specimens (Fig. 1). Subsequent inquiries of the donors revealed that 3 of the negative specimens were collected later than 24 hours after onset of illness. The normal limit of 15 cells per 100 fields was not exceeded with AF from passage of "normal" AF and with AF from uninoculated eggs. The

TABLE I. Serum Neutralization of Influenza Viruses. Detection by histiocyte count and by hemagglutination technics.

Virus	Without serum	+ antiserum for influenza A	A′	B
A	360/ 640*	0/ 0	177/320	140/320
A′	104/2560	224/ 640	1/ 0	143/640
B	192/ 160	312/1280	316/640	0/ 0

* No. of histiocytes per 100 fields/reciprocal of hemagglutination titer.

[‡] Stained with "Hansel" stain, Lide Laboratories, St. Louis, Mo.

TABLE II. Titration of Inoculums in Embryonated Eggs.

Agent	No. of histiocytes per 100 oil immersion fields 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Influenza A	—	200+/1280*	180/2560	266/2560	200/2560	0/ 0	—	—
" A′	—	200+/2560	237/2560	210/2560	288/2560	152/1280	102/320	0/0
" B	—	182 /1280	151/1260	154/ 640	3/ 0	—		
Mumps	46/160	47 / 160	3/ 0	12/ 0	—			

* Histiocyte count/hemagglutinin titer.

effect of nasal washings from the 11 convalescent patients who were available contrasted markedly with that of their specimens collected during the acute stage of illness (Fig. 2): none of the convalescent specimens engendered counts in excess of the normal limit. These were collected and processed by the same method as the acute specimens. Simultaneous inoculations into groups of eggs of untreated and of heated nasal washings indicated that the agent responsible for the high histiocyte count was thermolabile (Fig. 3).

Smears from allergic rhinitis tissues revealed numerous eosinophilic leukocytes, whereas smears from the common cold cases contained numerous epithelioid mononuclear cells, and varying numbers of neutrophilic leukocytes, the latter predominating during the later stages of illness. Nasal washings from 23 cases of acute allergic rhinitis failed to induce histiocytes in excess of the normal limit (Table III). Nasal washings collected during the same period from common cold patients showed in 7 of 8 instances increased numbers of histiocytes in inoculated eggs. (Table IV).

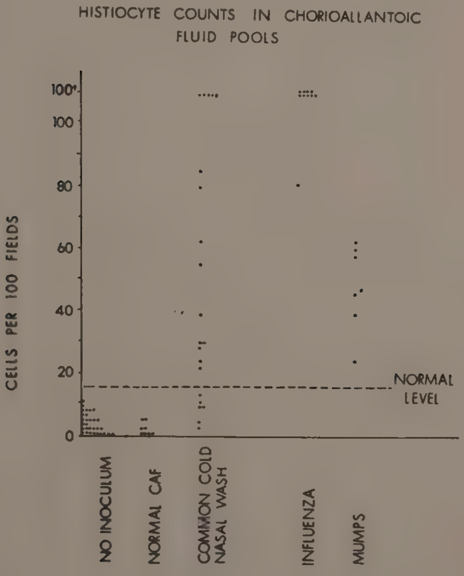

FIG. 1.

TABLE III. Histiocyte Response to Inoculums from 23 Patients with Allergic Rhinitis.*

Date nasal wash Collected	Inoculated	Histiocytes in AF/100 fields
9–13–55	9–16–55	11
10– 1	10– 5	4
1	5	3
13	14	5
14	14	4
14	14	8
14	14	3
14	18	0
14	18	10
14	25	6
24	26	10
24	26	5
21	26	7
24	26	4
27	11– 9	5
11– 1	2	4
4	9	15
4	9	4
4	9	3
8	9	3
8	9	4
8	9	4
1– 9–56	1–10–56	15

* Numerous eosinophilic leukocytes noted in smears of nasal effluent.

TABLE IV. Histiocyte Response to Inoculums from 8 Cases of Afebrile Rhinitis (Common Cold).

Date nasal wash Collected	Inoculated	Nasal smear	Histiocytes in AF/100 fields
10–24–55	10–26–55	Epithelial cells	64
3	11– 9	Mononuclear cells	62
11– 8	9	Rare eosinophiles	100+
11	16	Epithelial cells	17
18	18	No cells	30
21	23	Epithelial cells	70
12– 8	12– 9	*Idem*	75
1– 5–56	1–10–56	"	65

Passage of AF, from eggs in which high counts had been engendered with acute common cold specimens revealed high counts during the succeeding 5 and 6 passages. Thereafter the majority of cell counts declined abruptly to normal levels. Continued passage was accompanied by irregular rises and falls in cell counts. Serial passages of AF from uninoculated eggs induced no significant cell counts in AF. Hemagglutination could not be demonstrated in any of the passaged AF which originated from the acute common cold cases. Bacterial agents could not be demonstrated in any of the fluids by microscopic and by culture technics.

Discussion. The cytological content of AF appears to reflect viral activity, as has been demonstrated in parallel studies for hemagglutinin and histiocyte counts in influenza and mumps-infected allantoic fluids. Both technics were equally sensitive. A similar interpretation might be ascribed to this cytological response by inoculums from acute common cold cases. When the acute nasal washings were treated at 61°C for 30 minutes, this response was negated. Furthermore, the histiocyte response did not occur with nasal washings collected from the same patients during convalescence. The agent responsible for the histiocyte response may not be the etiological agent of common cold infection; however, its chronological association with the syndrome is suggestive of that possibility. If latent viral agents were responsible for the histiocyte response, they should have been demonstrable in the convalescent nasal washings.

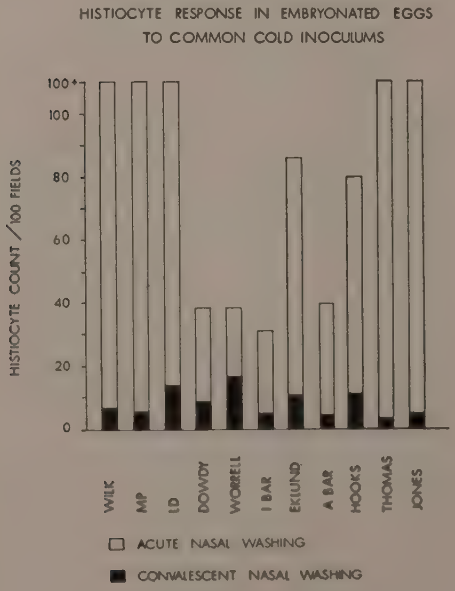

FIG. 2.

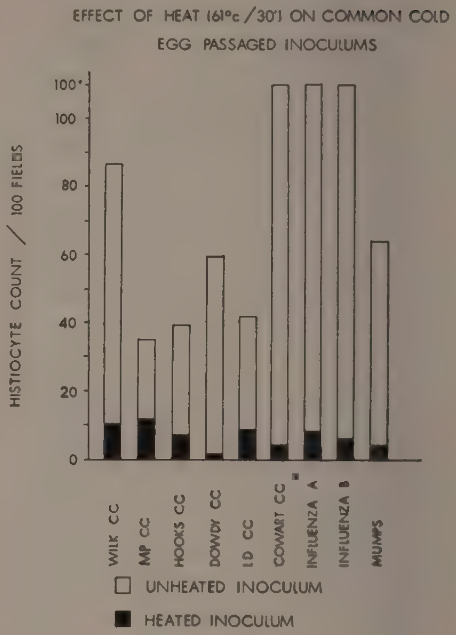

FIG. 3.

The significance of these results is further supported by the failure of nasal washing from allergic rhinitis cases to engender a significant histiocyte response in AF. Such allergic manifestations were often clinically indistinguishable from the acute cases of common cold. The conditions under which the specimens were collected had no influence on the results, since all specimens were collected and stored under the same conditions (Tables III and IV). The agent(s) do not appear to be influenza-like, since egg passaged material failed to agglutinate chicken erythrocytes.

The histiocyte response is associated with inoculums collected during the acute stage of common cold illness. Although this is a non-specific response, it might be rendered significant through inhibition by specific antiserums as was demonstrated with influenza virus (Table I). Neutralizing antiserums, prepared in chickens with several strains of common cold agent thus far studied, have demonstrated more than one antigenic strain.

It would be premature to say that even one common cold agent has been adapted to or propagated in the tissues of the embryonated egg. The decline of cytological response with passage gives no support to this proposition. Further studies on optimal passage interval and on tissue susceptibility may provide a means of adapting such agents to avian tissues and the process may be revealed through the cytological response in the AF of inoculated eggs. This would provide a technical advantage over the use of human volunteers for the detection of virus.

Summary. (1) Influenza and mumps viruses induced increased numbers of histiocytes in the allantoic fluids of embryonated eggs. The significance of this response was enhanced by coincident hemagglutinin production and by an interference effect on both responses by specific antiserums. (2) Nasal washings from acute common cold (afebrile rhinitis) patients engendered increased numbers of histiocytes in the allantoic fluid of embryonated eggs. The agent(s) responsible for this cytological response were thermolabile, were not inhibited by penicillin and streptomycin, and produced no detectable growth in bacteriological media. The agent(s) were not demonstrable in nasal washings collected from the same patients during convalescence. (3) Nasal washings from cases of acute allergic rhinitis did not induce increased numbers of histiocytes in the allantoic fluid of inoculated eggs.

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Inhibition by Aminopyrine of Adrenocortical Activation Caused by Pyrogenic Reaction.[*] (22291)

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Like a number of other stressful stimuli, typhoid vaccine has been shown to cause stimulation of the pituitary-adrenal axis in rats (1). In human subjects, Arendshorst and Falls(2) showed the occurrence of eosinopenia following typhoid vaccine administration, and Rosen(3) and Conn, *et al.*(4) demonstrated increases in urinary 17-ketosteroid and "corticoid" excretion after injection of this material. Bliss and his co-workers(5) found that milder febrile stimuli, produced by bacterial pyrogen (Piromen®), caused moderate rises in the levels of plasma 17-OH-corticosteroids.

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It seemed that this adrenocortical response to a stimulus might afford a means of studying some of the factors involved in activation of the pituitary-adrenal system. In this study, an attempt was made to learn whether the adrenocortical stimulation depended entirely on the febrile response to typhoid vaccine, or could be produced in the absence of fever. This approach appeared to be of particular interest in view of the studies of Bradley, *et al.*(6), who found that an antipyretic agent inhibited the febrile response to typhoid vaccine, but did not prevent certain renal hemodynamic changes characteristic of the pyrogenic reaction.

Materials and methods. The patients included in this study were 17 adult men and women, in good general health, who were admitted to the Institute of Ophthalmology of the Presbyterian Hospital for treatment of various inflammatory disorders of the eye. Typhoid vaccine (obtained from the Department of Health, City of New York—1 ml contains 1,000 million killed *S. typhosa* organisms, 250 million killed *S. paratyphi* A, and 250 million killed *S. paratyphi* B organisms) was diluted with saline and injected in a standardized manner. 5 to 10 million *S. typhosa* organisms (plus 1.25 to 2.5 million of both *S. paratyphi* A and B) were administered in a single intravenous injection, followed in 2 hours by a similar dose. Rectal temperatures were taken at hourly intervals. Adrenocortical response was assessed by measuring changes in plasma 17-OH-corticosteroid levels, determined according to the method of Silber and Porter(7) as previously described(8). Control blood specimens were obtained, and additional specimens were drawn at intervals following administration of typhoid vaccine (Table I). No rise in plasma 17-OH-corticosteroid level was regarded as significant unless values exceeded the upper limit of normal. Normal limits in this laboratory are 4-28 μg per 100 ml plasma. In 7 of the patients, the anti-pyretic, aminopyrine was given over a 24-hour period in a dose of 0.6 g every 4 hours. Immediately following the last dose of aminopyrine, typhoid vaccine was administered according to the schedule outlined above, with blood samples being obtained in the same manner.

TABLE I. Effect of Intravenous Typhoid Vaccine on Levels of Plasma 17-OH-Corticosteroids. Time in hr after 2nd vaccine injection.

Patient	Max temp., °F	Plasma 17-OH-corticosteroids, γ %			
		Control	2 hr	4 hr	6 hr
1	103.8	18	43	34	
	103.8	28		42	40
2	104.8	15	59	30	
	102.4	13	32		
3	103.8	17	40	35	16
	103.4	22		32	
4	102.6	20	40		
5	102.8	13		30	
6	105.6	14	53	36	
7	104.2	8	28	29	
	105.6	13	46	34	16
8	103.8	7	32		
9	103.6	17	36		42
	104.0	19	37		13
	104.8	18		35	
10	103.6	17		35	
	102.6	24			24
11	104.0	20	43	45	
12	102.6	18	33	22	
	102.4	14	38		
13	104.4	17	36		
	103.4	26		27	
14	102.6	16	36		
15	102.4	14	29	17	

Results. 1. *Effect of typhoid vaccine alone.* In 15 patients, typhoid therapy was given on 24 separate occasions. In all 24 instances, there was a sharp rise in temperature to 102.4-105.6°F, occurring within 2 to 5 hours after the second dose of vaccine. The control 17-OH-corticosteroid values fell within the normal range (Table I). In 22 of the 24 experiments, plasma 17-OH-corticosteroid levels rose, within 2 to 6 hours after the second dose, to values above the normal which ranged from 29 to 59 μg per 100 ml plasma. These levels were comparable to those attained in normal subjects after intravenous infusion of 25 mg ACTH(9). In 4 of the patients, in whom studies were made of diurnal variations in levels of circulating 17-OH-corticosteroids, such abnormally high values were not found. In these patients, corticosteroid levels fluctuated within the normal range. In 4 additional instances, typhoid vaccine failed to produce a rise in temperature above 101°F, and the febrile response was delayed. In these cases, plasma corticosteroid levels did not rise above the upper limit of normal.

2. *Effect of aminopyrine.* In all 7 patients treated with aminopyrine, injection of typhoid vaccine failed to cause pyrexia above 100.4°F. In 6 of the patients, there were no significant rises in plasma 17-OH-corticosteroid levels, values remaining within normal limits (Table II). In the seventh (patient #8, Table II), plasma 17-OH-corticosteroid levels rose from 20 to 28 μg per 100 ml, the upper limit of normal. It was noteworthy that in all 7 subjects, constitutional symptoms (malaise, weakness, nausea, chilly sensations) occurred despite the absence of fever. Failure of plasma 17-OH-corticosteroid levels to rise in the aminopyrine-treated subjects did not seem attributable to refractoriness to the pyrogenic effect of the vaccine. As can be seen in Fig. 1, repeat administration of comparable doses of vaccine in the 3 patients restudied without aminopyrine, again caused rises in temperature, associated with an increase in plasma 17-OH-corticosteroid values.

3. *Effect of central nervous system depressants.* (a) Barbiturate. In a single subject, 1 g of Amobarbital sodium (Na amytal) was administered in divided doses before and during standard vaccine injection. The temperature rose to 105.6°F within 3 hours after the second dose of vaccine. Plasma 17-OH-corticosteroid rise was not inhibited, rising from a control level of 13, to 43 μg per 100 ml.

TABLE II. Effect of Aminopyrine on Plasma 17-OH-Corticosteroid Response to Intravenous Typhoid Vaccine. Time in hr after 2nd vaccine injection. (Patient numbers correspond to those in Table I.)

Patient	Max temp., °F	Plasma 17-OH-corticosteroids, γ % Control	2 hr	4 hr	6 hr
1	99.4	25	24		13
2	99.6	14	17		11
3	100.4	24	22	24	
8	99.6	20	28		
14	98.6	14	19		
16	99.0	27	27		
17	98.4	28	20		

(b) 10-(γ-dimethylaminopropyl)-2-chlorophenothiazine (Chlorpromazine). This broadly-acting agent, chosen because of its depressant effect on hypothalamic centers(10), was administered to 8 patients in doses up to 250 mg, before and during typhoid vaccine therapy. In 6 instances, plasma 17-OH-corticosteroid rises occurred in a manner comparable to that shown in Table I. In 2 cases, corticosteroid rises did not occur, but in both instances, febrile response was delayed and was of only moderate degree.

4. *Controls.* (a) To rule out the possibility that the aminopyrine suppression of plasma corticosteroid rise could be due to blockade of adrenocortical response to endogenous ACTH, a single normal male was treated for 24 hours with aminopyrine (0.6 g every 4 hours), and then subjected to a standardized ACTH test. Plasma 17-OH-corticosteroid levels rose from 10 to 45 μg per 100 ml, a response well within the limits of normal(9). (b) To exclude a direct inhibitory effect of aminopyrine on pituitary release of ACTH, a normal subject was given an insulin tolerance test (0.15 unit of standard insulin per kilogram body weight administered intravenously). Plasma 17-OH-corticosteroid levels rose from 20 to 28 μg per 100 ml(5). After treatment for 24 hours with aminopyrine, 3.6 g, repeat insulin administration was again associated with a rise in plasma corticosteroid value, from 6 to 30 μg per 100 ml. (On 2 different days, diurnal variation in plasma corticosteroid levels in this patient showed a definite fall in values.)

Discussion. The rise of plasma 17-OH-corticosteroids to levels above the normal following typhoid vaccine injection confirms the fact that this agent is capable of producing adrenocortical activation in the human subject(2-5). Blockade of the febrile response to the vaccine by aminopyrine inhibits this plasma corticosteroid increase. The inference is that the fever *per se* is the essential factor in bringing about activation of the pituitary-adrenal system. Presumably, other

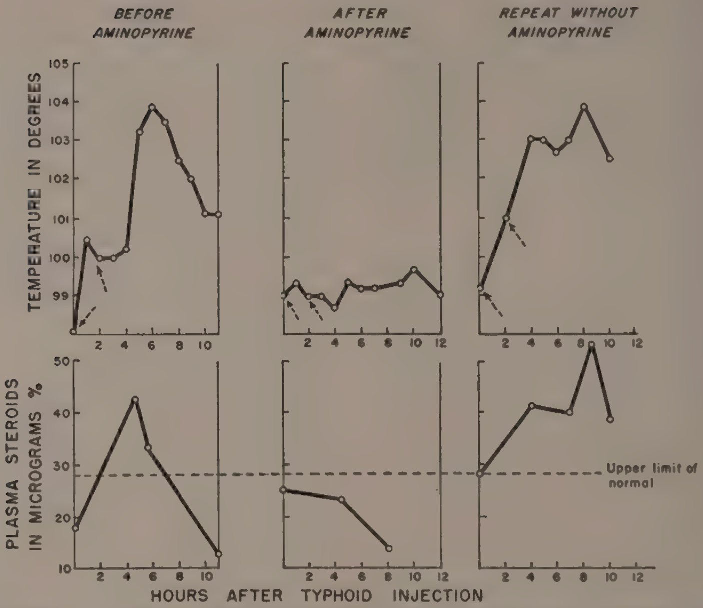

FIG. 1. Febrile and plasma 17-OH-corticosteroid response to intravenous typhoid vaccine injection (patient #1, Table I). The 3 series of vaccine injections were performed at intervals of 3 days. Arrows indicate times of injection of vaccine.

noxious effects of the vaccine which are not prevented by an antipyretic agent(6), are not effective in activating the adrenal cortex, as measured by this index of response. Furthermore, other central nervous system depressants, in the doses used, failed to inhibit plasma corticosteroid rises, unless the febrile response was also partially blocked.

The inhibitory effect of aminopyrine did not appear to act directly on the pituitary-adrenal system. The drug did not interfere with the adrenocortical response to administered ACTH, nor did it inhibit the pituitary-adrenal reaction to a non-febrile stimulus (insulin hypoglycemia).

Summary. Intravenous administration of typhoid vaccine to human subjects produced adrenocortical activation, as measured by increases in levels of circulating 17-OH-corticosteroids. Suppression of the pyrogenic effect of the vaccine by aminopyrine prevented adrenocortical response, without blocking certain other systemic reactions.

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Further Studies of Interrelationship Between Xanthine Oxidase and Influenzal Pneumonia in Mice.* (22292)

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It has been previously reported(1) that an increase in xanthine oxidase occurs in the lungs of mice infected with PR8, Type A influenza virus. In order to define further the interrelationship between the increased levels of enzyme and virus proliferation additional experiments have been carried out. Levels of the *in vitro* activity of xanthine oxidase in mouse lung infected with influenza virus have been compared with those of the same tissue either infected or injured with: (a) Nigg pneumonitis virus, (b) Newcastle disease virus, (c) *Klebsiella pneumoniae,* and (d) toxic chemicals, fats, Friedlander's polysaccharide and leukotaxine. The results indicate that although the increase in xanthine oxidase is not directly related to influenza virus proliferation *per se,* the enzyme may be concerned in some way with the pneumonic process in mouse lung.

Methods. Four to 6 weeks old albino Swiss mice of the Webster strain were employed. Xanthine oxidase assays on homogenized mouse lung tissue were carried out in a conventional Warburg apparatus as described previously(1). The values reported represent the oxygen-uptake, stimulated by addition of substrate, of 20 mg tissue (dry weight equivalent) per 20 minutes; in each experiment the oxygen uptake representing the endogenous respiration has been subtracted from the recorded values. Hemagglutination titrations were performed employing a pattern end point (2). In determining the virus infectivity titers, equal volumes of a sample of homogenate from all the animals assayed at a given time were pooled, serial 10-fold dilutions prepared and 4, 10-day chick embryos inoculated with 0.2 ml of each dilution. After 48 hours incubation at 35°C, samples of allantoic fluid were removed and tested for the presence of virus by the spot plate hemagglutination technic(3). The ID_{50} titers were calculated according to the method of Reed and Muench (4). The lungs from each animal were examined and the pulmonary consolidation scored according to the method of Horsfall (5).

Results. The results of an experiment with a mouse adapted strain of PR8, Type A influenza virus are presented in Table I. Mice were infected via the intranasal route with 4,000 LD_{50} doses of stock virus. The day-to-day change in enzyme level together with the change in virus level as determined by hemagglutination titers for chick-embryos were determined on combined aliquots of tissue from each day's experiment; these results are directly comparable to the recorded averages of enzyme activity. Levels of enzyme activity are roughly paralleled by virus titers.

Consolidation is somewhat slower than is the increase in enzyme activity in developing; the presence of gross lesions is not essential for increased xanthine oxidase activity although the maximal levels of enzyme are found in partially consolidated tissue. A lethal dose of virus was employed in this experiment and at the end of 100 hours all the animals were dead. When sub-lethal amounts of virus were employed comparable results were obtained, *i.e.*, the development of enzyme levels and virus titers roughly paralleled each other although the levels reached were lower and slower in developing than in lethally infected mice.

An experiment showing the changes in xanthine oxidase in mouse lung following infection with Nigg pneumonitis virus (Greb

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† The author wishes to thank Dr. A. F. Rasmussen, Jr., for valuable suggestions concerning this work.

TABLE I. Correlation of Changes in Virus Concentration and in Xanthine Oxidase Activity in Mouse Lung Infected with PR8 Influenza Virus.

Time	Group 1*			Group 2			Group 3			Avg		
(hr)†	E.A.‡	H.T.§	C.‖	E.A.	H.T.	C.	E.A.	H.T.	C.	E.A.	H.T.	EID_{50}
0	2.3			2.5			2.3			2.4		
24	6.9	160	0 0	5.3	160	+ 0	4.9	160	0 0	5.7	160	$10^{-7.75}$
48	8.5	320	0 0	7.2	320	+ +	5.9	320	0 +	7.2	320	$10^{-8.5}$
72	13.2	320	2+ +	9.8	320	4+ 2+	8.0	160	3+ 2+	10.3	266	10^{-9}
96	12.0	80	3+ +	4.8	320	3+ 2+	3.9	80	2+ +	6.9	240	$10^{-7.5}$

* Group of 10 mice, 2 chosen at random and pooled for examination at indicated time.

† No. of hours between initial infection and titration.

‡ Enzyme activity: μl O_2 uptake/20 min./20 mg dry lung. Substrate: 10 μM hypoxanthine/flask.

§ Hemagglutinin titer: Highest dilution of virus showing complete hemagglutination.

‖ Amount of consolidation determined by gross macroscopic examination and scored on a 0-5 point basis.

EID_{50}—50% embryo infectivity end point.

strain) is recorded in Table II. The animals were infected intranasally with 10-100 lethal doses of virus. Although the titer of virus in the tissue examined for enzyme activity was not determined, the presence of virus was established by mouse inoculation. In comparison to the PR8 infection, a somewhat greater level of enzyme activity was reached on each successive day reaching a peak value of 12.4 on the fourth day. Only 3 of the animals survived to the fifth day; the value obtained for the enzyme activity of these lungs was 11.4 which may have indicated a leveling off of activity.

Table III shows the changes in xanthine oxidase in mouse lung following the administration of Newcastle disease virus (NDV). The mice were given 0.05 ml of infected allantoic fluid (ID_{50} titer for eggs of $10^{-7.6}$) by the intranasal route. There was a sharp rise in enzyme activity almost reaching a peak level within 24 hours and remaining relatively constant through the fourth day, while the macroscopic consolidation developed gradually over a three day period.

For comparative purposes, studies were made of the effect of a pulmonary bacterial infection on xanthine oxidase. Virulent *Klebsiella pneumoniae,* when introduced via the intranasal route, produced a rapidly fatal pneumonitis. Within 48 hours the lungs were almost completely consolidated, and the ani-

TABLE II. Change in Levels of Xanthine Oxidase Activity in Mouse Lung after Infection with NIGG Pneumonitis Virus.

Time	Group 1*		Group 2		Group 3		
(hr)†	E.A.‡	C.§	E.A.	C.	E.A.	C.	Avg E.A.
0	2.6		2.0		2.1		2.2
24	6.4	+ —	5.6	+ +	3.8	+ —	5.26
48	11.4	4+ +	8.1	3+ 2+	8.0	3+ +	9.16
72	11.0	4+ 2+	10.7	3+ 3+	10.0	3+ 3+	10.56
96	14.6	3+ 3+	11.8	4+ 3+	10.9	4+ 3+	12.43

* Group of 10 mice, 2 chosen at random and pooled for examination at indicated time.

† No. of hours between initial injection and assay.

‡ Enzyme activity: μl O_2/20 min./20 mg dry lung. Substrate: 10 μM hypoxanthine/flask.

§ Consolidation.

TABLE III. Change in Levels of Xanthine Oxidase Activity in Mouse Lung after Administration of Newcastle Disease Virus.

Time	Group 1*		Group 2		Group 3		
(hr)†	E.A.‡	C.§	E.A.	C.	E.A.	C.	Avg E.A.
0	2.6		2.0		2.1		2.2
24	10.0	0 +	8.3	0 +	6.5	+ +	8.26
48	9.0	4+ 3+	8.1	+ 0	7.7	+ +	8.26
72	11.8	5+ 0	8.9	2+ +	7.3	4+ 4+	9.33
96	11.6	+ +	9.4	5+ 3+	7.0	+ +	9.33

* Group of 10 mice, 2 chosen at random and pooled for examination at indicated times.

† No. of hours between initial inoculation and assay.

‡ Enzyme activity: μl O_2/20 min./20 mg dry lung. Substrate: 10 μM hypoxanthine/flask.

§ Consolidation.

mals seldom survived a lethal dose beyond this time. Enzyme assays showed that the level of xanthine oxidase was increased concomitant with the progressing *K. pneumoniae* infection: Values of 4.0, 5.4, and 6.5 (μl O_2 uptake/20 minutes/20 mg dry lung) at 24 hours and 5.4, 5.6, and 6.7 at 48 hours were obtained in a typical experiment. These enzyme levels, when compared with the levels of lungs infected with influenza virus are comparable at the same time intervals; however, the maximal levels of enzyme in the influenza lungs at the end of 96 hours, when the lesions are more comparable, are about two times these values. That the enzyme activity was not due to the bacteria present in the homogenate was shown by testing the organisms for xanthine oxidase activity. The strain of *K. pneumoniae* employed in these experiments showed almost no activity, and efforts to produce an "adaptive" type of this enzyme were not successful. The results indicate that infection of mouse lung with *K. pneumoniae* produces a severe pneumonitis and gives rise to an increase in xanthine oxidase activity.

The following compounds were employed in attempts to produce a sterile pneumonia in mice: dilute ammonia, Friedlander's polysaccharide, olive oil, starch, heparin, leukotaxine, serum and turpentine. Intranasal, intratracheal and intrapleural routes of inoculation were tried. In no instance was a pneumonic process produced equal in magnitude to that produced by virus or *K. pneumoniae*. In 10-25% of the animals one lobe, or perhaps 2 lobes, showed some consolidation; xanthine oxidase was not increased in these lungs. The most successful procedure was that of intrapleural injection of turpentine; at the end of 96 hours some of these lungs showed a moderate amount of consolidation and a concomitant increase in xanthine oxidase of about the same magnitude as the 48-hour influenza lung. Therefore, it is apparent that the increase in xanthine oxidase can be elicited in the absence of an infectious process and may be the result of an inflammatory reaction.

In order to determine if basic similarities existed in the pathology of the lesions produced by these various agents, microscopic sections were examined. Sections of influenza lungs showed diffuse interstitial pneumonitis with a mononuclear infiltrate causing a thickening of septal walls. Necrosis and desquamation of the epithelial lining of the bronchi and bronchioles were noted. The lesions produced by NDV were very similar in character although less severe in degree. Sections of the lesions produced by *K. pneumoniae* and by Nigg pneumonitis virus showed severe bronchopneumonia with diffuse areas of cellular infiltrate, predominantly polymorphonuclear leucocytes. There was associated distortion and destruction of lung architecture. The mucosae and walls of the bronchi and bronchioli appeared to be relatively uninvolved although the lumina were plugged with exudate. A correlation between lesions with characteristic infiltrates and xanthine oxidase activity was not found.

Discussion. In mouse lungs infected with the viruses of influenza and Nigg pneumonitis, a fundamental relationship between purine catabolism and virus synthesis is suggested by an increase in xanthine oxidase activity which occurs during the phase of rapid increase in virus concentration and precedes the development of maximal macroscopic and microscopic lesions. The fact that a similar change occurs after NDV which does not multiply in mouse lung(6,7) might contradict such a hypothetical relationship. On the other hand, it is conceivable that a change in purine metabolism is initiated by invasion of the host cell by viral particles whether or not subsequent multiplication of the virus takes place. Comparative studies of other types of injury to mouse lung indicate that infection with *K. pneumoniae,* and the intrapleural injection of turpentine also result in significant increases in xanthine oxidase activity. These latter findings negate the intriguing possibility that xanthine oxidase is concerned with a phase of nucleic acid metabolism intimately related to viral synthesis. The fact that a variety of microorganisms capable of producing severe and rapidly fatal pneumonias all elicit a rapid increase in xanthine oxidase activity remains of some interest. One may ask whether or not this represents a biochemical disturbance of host-cell metabolism which contributes to the over-all disease process.

Conclusion. Previous observations that xanthine oxidase increases in mouse lung concomitant with influenza virus infection have been confirmed and extended. Comparable increases in xanthine oxidase also occur in murine pneumonitis produced by Nigg pneumonitis virus and by NDV. Comparative studies with other types of infection and injury indicate that infection with *K. pneumoniae* and the intrapleural injection of turpentine also result in significant increases in xanthine oxidase activity in mouse lung thus suggesting that an increase in the level of enzyme is closely associated with injury and death to lung tissue.

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Fluorescent Antibody Studies of Demyelination in Canine Distemper.* (22293)

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The literature on central nervous system lesions in canine distemper has been adequately reviewed by Winqvist(1). Demyelination and glial cell proliferation are the outstanding changes while vascular hyperplasia and perivascular infiltrations occur less frequently. The cerebellum, pons and medulla are the principal sites of involvement. No attempt has been made to correlate the demyelination with localization of the virus. This report describes the demyelination process as it occurs in the cerebellum, and by means of the fluorescent antibody technic(2) to demonstrate the sites of viral concentration.

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† The author is indebted to Dr. J. R. Gorham for performing the *in ovo* neutralization tests, to Mrs. Lillian Frazier for the special stains, and to Mr. L. M. Hardaker for photomicrographs.

Methods. Tissues for histopathologic study were obtained at autopsy from naturally occurring cases of distemper. All the dogs had typical distemper inclusion bodies either in urinary bladder epithelium or in astrocytes of the cerebellum. Blocks of cerebellum were fixed in 10% formalin, Zenker's and ammonium bromide-formalin mixtures. Hematoxylin and eosin, Weil, Bodian protargol, pyridine silver, cresyl violet, Feulgen Mallory phosphotungstic acid, Cajal gold sublimate and oil red O. B. staining methods were employed. The fluorescent antibody technic is based on the fact that fluorescein can be conjugated with antibody molecules without loss of activity. When tissue sections containing antigen specific for the antibody solution are flooded with such fluorescein-antibody conjugate, the antibody combines with the antigen. After the excess conjugate is rinsed off, the antibody is visible under a fluorescence microscope by virtue of its fluorescent yellow-green label. *Hyperimmune canine distemper* serum was conjugated to fluorescein isocyanate according to the method of Coons and Kaplan(2). Conjugates with greater antibody titer were obtained with freshly prepared isocyanate than with isocyanate which had been previously prepared and stored. Tissues for fluorescent staining were obtained from clinical cases of distemper immediately after death. Blocks of cerebellum were placed in test tubes and quickly frozen by immersion in a bath of dry ice and alcohol. They were stored at –20°C until needed. Frozen sections were cut from these blocks at 15μ and mounted directly on slides without floating on water. Sections were dried on the slides by placing them in an incubator at 37°C for one hour. No chemical fixatives were employed. The sections were stained 30 minutes in fluorescein-antibody conjugate and then rinsed off with buffered saline, pH 9, and gently washed in fresh buffered saline for 10 minutes. Slides were wiped dry except for the section and mounted in glycerol-saline(9:1). Sections were examined in the dark with a fluorescence microscope (3). The light source was a Leitz, 15 ampere, carbon arc. Specific fluorescence appeared brilliantly yellow-green against a dark background. Fluorescent structures were identified by floating off the coverslip of the slide, counterstaining with a color dye and examining the same field under white light (3).

The *conjugate* used in this study was tested for antibody content by means of an *in ovo* neutralization test(4). The first sample had 1:630 titer and its deactivated blank, which was heated to 65°C for one hour, tested 1:250. The second sample had a titer of 1:160 and its blank, which was heated to 95°C for one hour, was negative. The difference in titers between the two samples was due to variations in quantity of virus used in the test and did not indicate a drop in antibody content in the conjugate(4). Conjugate was tested for specificity by staining smears of urinary bladder epithelium from dogs with distemper. Inclusion bodies in bladder epithelium show specific fluorescence when stained with distemper fluorescent antibody(3). *Slide controls* consisted of 1) staining brain tissues of normal 7-week-old puppies in the same manner as tissues from infected dogs, and 2) inhibiting the antigen in the tissue sections by treating with unconjugated distemper antiserum for 30 minutes prior to staining with conjugate.

Results. Weil stains of the cerebellum showed patchy areas of demyelination in the medullary white matter (Fig. 1). Early lesions appeared as empty spaces or holes in the white matter where there had been focal loss of myelin in 4 or 5 fibers. With loss of myelin, edema fluid collected in these foci, forced demyelinated axis cylinders to the periphery and compressed adjacent myelinated fibers.

When many holes formed in an area they coalesced into larger, spongy areas of demyelination (*Lückenfelder*). At the same time glial proliferation was noted. This occurred in response to disintegrated myelin for the majority of cells were microglial elements containing droplets of myelin (Fig. 2). Ameboid astrocytes were also present in the demyelinated areas. Glial scars were never observed.

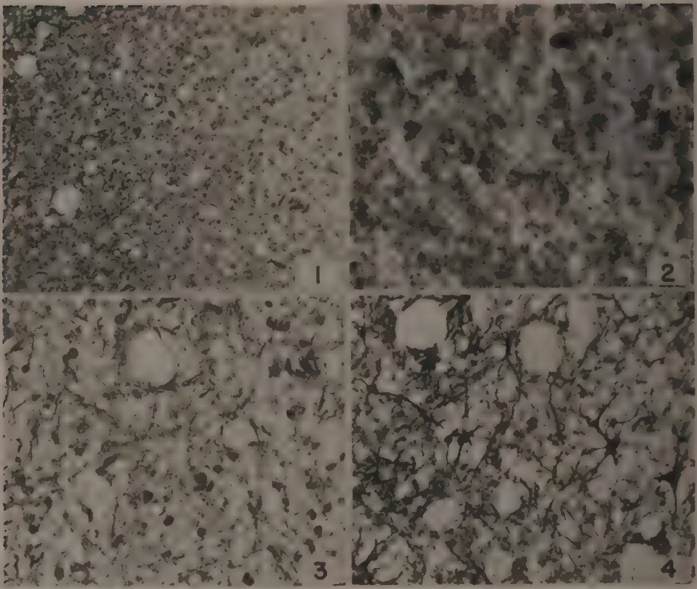


FIG. 1. Demyelination of cerebellar white matter in canine distemper. Weil stain. × 225.

FIG. 2. Microglial phagocytes filled with myelin droplets in an area of demyelination. Oil red O. B. stain. × 450.

FIG. 3. Axis cylinders in area of demyelination. Fibers show separation but no loss in continuity. Bodian protargol. × 450.

FIG. 4. Astrocytes in area of demyelination. Processes are intact although pushed apart by edema fluid. Cajal gold sublimate. × 450.

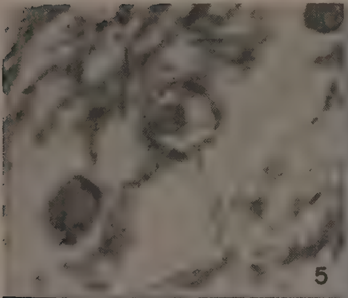
Axis cylinders were seldom involved although cylinders passing through severely demyelinated areas were stained poorly and separated by the edema fluid (Fig. 3).

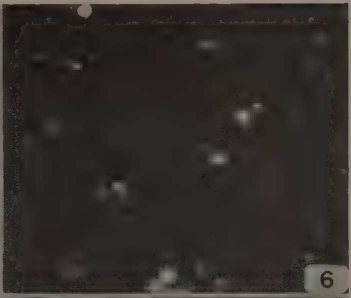
The majority of fibrous and ameboid astrocytes exhibited degenerative nuclear changes. This was not limited to astrocytes in demyelinated areas, but was also found in astrocytes at a distance. Astrocytic processes were not involved (Fig. 4). The nuclei of the degenerated astrocytes appeared swollen and pale and often contained one or more acidophilic inclusion bodies (Fig. 5). Nuclear chromatin was marginated in these cells. Feulgen reactions were weakly positive in the degenerated astrocyte nuclei and negative in the inclusion bodies. Oligodendroglia showed some nuclear degeneration, but fewer cells were involved. Neurons were normal except for those in severely demyelinated areas which showed chromatolysis.

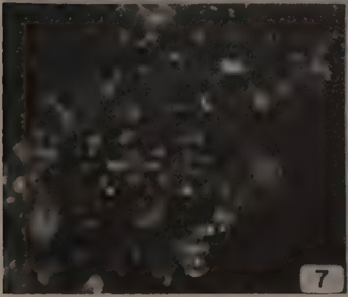
Inflammatory cells were usually absent, but in severely demyelinated areas perivascular inflammatory cells were sometimes noted. These consisted of plasma cells, lymphocytes and macrophages and were restricted to the Virchow-Robin space. A few sections exhibited proliferation of blood vessels in advanced areas of demyelination.

Sections stained with fluorescein-antibody conjugate showed specific fluorescence in the cerebellar white matter (Fig. 6). Fluorescence in the cerebellar medullary lamellae was especially remarkable because of the contrast of the non-fluorescent cortex (Fig. 7). Specific yellow-green fluorescence appeared to be localized principally in astrocyte nuclei although it was difficult to tell astrocytes from other glial cells. It was impossible to tell whether or not inclusion bodies were fluorescing. Fluorescence was not limited to cells in demyelinated areas, but involved cells throughout the white matter. Myelin sheaths, neurons and axis cylinders showed no specific fluorescence.

Control slides reacted negatively. Sections







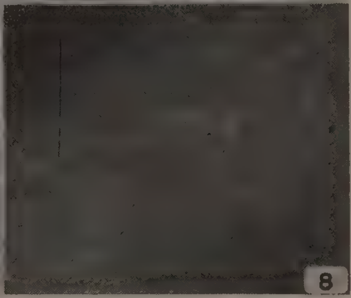


FIG. 5. In center is an inclusion body in nucleus of an astrocyte. Hematoxylin and eosin. × 600.

FIG. 6. Localization of viral antigen in glial nuclei of cerebellar white matter. Fluorescent antibody technic. × 450.

FIG. 7. Cerebellar lamella of dog with distemper showing fluorescent staining of glial cells. Note absence of fluorescence in adjacent cortex. Fluorescent antibody technic. × 400.

FIG. 8. Same area as in Fig. 7 from a normal dog. This section was treated identically. Note absence of specific fluorescence in cerebellar lamella. Fluorescent antibody technic. × 450.

from normal dogs showed no specific fluorescence (Fig. 8). In pathological specimens staining was markedly reduced in tissues treated with normal antiserum before fluorescent antibody exposure.

Discussion. Demyelination has seldom been listed among the lesions produced by viruses infecting the nervous system. Viruses have not been considered seriously as possible etiological agents of the various demyelinating maladies. There is no experimental support for the belief that the post-vaccinal type of encephalomyelitis results from direct action of the virus(5). In the case of nervous distemper, however, recent studies point to the distemper virus as the agent responsible for the demyelination and the presence of nuclear inclusions in the glial cells is evidence of the presence of the virus(6). However, the mechanism of demyelination has not been explained.

Fluorescent antibody studies of distemper indicate that neuroglial nuclei are invaded by the virus and that the astroglia are primarily involved. There is good correlation between astrocytic nuclear degeneration as observed by conventional staining methods and localization of viral antigen as demonstrated with fluorescent antibody. Demyelination in distemper does not appear to be associated directly with neuronal or axis cylinder degeneration as these structures do not show specific fluorescent staining and exhibit few degenerative changes. It appears that demyelination in this disease is associated with viral invasion and degeneration of glia, especially astroglia although this relationship cannot be explained. Other than the possible association of oligodendroglia with the production and maintenance of myelin(7), no other relationship between glial cells and myelination is known.

When sufficient myelin is broken down, microglial phagocytes invade the area and ameboid astrocytes are evident. Perivascular inflammatory reaction is not a constant finding in this disease, but when it occurs it is probably a secondary reaction in response to disintegration of myelin(8).

Summary. Fluorescent antibody studies show that the virus of canine distemper is localized in astrocytic and possibly other glial nuclei. The astrocytes exhibit degenerative changes consistent with viral invasion. Astrocytic involvement appears to be followed by demyelination although this causative relationship cannot be explained.

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Monolayer Cultures of Trypsinized Monkey Kidney Cells in Synthetic Medium. Application to Poliovirus Synthesis.* (22294)

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The outgrowth of monkey kidney cells from suspensions of trypsinized tissue has always been found to require substantial amounts of serum in the medium. The necessity for adding serum has precluded many kinds of studies and has restricted the kind of cultures available for study. A simple synthetic medium is described which, in the absence of any added protein, supports outgrowth of monkey kidney cell suspensions. After outgrowth in this medium, the cells support synthesis of poliovirus and may be used for physiological studies under defined conditions in the absence of serum.

Methods. Cell suspensions. Trypsinization of kidneys taken from rhesus monkeys was done by a modification(1) of the Youngner procedure(2). The cells were harvested from the trypsin fluid by centrifugation at 200 rpm for 30 minutes, resuspended in 500 x their volume of Basic Salt #2 without bicarbonate (as described in Table I), and recentrifuged. The washed cells, resuspended in 100-200 ml of synthetic medium, were filtered through 2 layers of cheese cloth. A count of those cells showing both nuclei and attached cytoplasm was made by staining with 0.1% crystal violet-0.1 M citric acid, and the suspensions were diluted to 350,000 cells per ml. Amounts of 0.5 ml were seeded into 16 x 150 mm test tubes, 7 ml into 2 oz and 12 ml into 4 oz prescription bottles, and 80-100 ml into Roux flasks. These amounts are also in the optimal range for cells suspended in the medium which contains serum (1). In experiments where outgrowth in the synthetic medium was to be compared to outgrowth in a serum-containing medium, the suspension in PBS was divided into 2 portions and the second half after recentrifuging was diluted into the serum-containing medium. The serum-containing, "complete" medium (called "M") used for comparison was the 0.5% lactalbumin hydrolysate-2% calf serum medium in Hanks' salt solution which has been used extensively in this laboratory(3).

Virus titrations. These were made by the

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* This investigation was aided by grant from National Foundation for Infantile Paralysis and by research contract with Division of Biologics Standards of National Institutes of Health, Public Health Service.

TABLE I. Synthetic Medium (SM-1) for Outgrowth of Monkey Kidney Cell Suspensions.

Basic salt sol. (B.S. #2)*	g/l	Trace elements†	$M \times 10^{-6}/l$
NaCl	8.0	$ZnSO_4$	3
KCl	.4	$Fe(NO_3)_3$	2
$MgSO_4$ 7 H_2O	.1	$CoCl_2$	.2
MgCl 6 H_2O	.1	$MnCl_2$	.5
Na_2HPO_4	.08	$CuSO_4$	.4
NaH_2PO_4	.02		
$CaCl_2$	.42		
$NaHCO_3$	.78		
Glucose	2.0		

Organic supplements‡	mg/l	Supplementary buffer§
l-cysteine • HCl	30	Tris (hydroxymethyl aminomethane)
l-isoleucine	60	
d-ribose	30	8×10^{-3} M, pH 7.6

* Made in 10 × concentrated, combined stock without $CaCl_2$ and $NaHCO_3$. Sterilized by filtration. $CaCl_2$ is made at 100 × concentrated; $NaHCO_3$ is made at 100 × concentrated. Glucose in 20% stock solution, sterilized by filtration.

† Held as separate stocks, 0.05% concentrations. Sterilized by filtration.

‡ Stocks 100 × concentrated. Sterilized by filtration. May be held as combined stock 40 × concentrated in H_2O.

§ Conveniently stored as 2 M stock adjusted to pH 7.6 with HCl. May be sterilized by autoclaving at 15 lb for 15 min.

Dulbecco-Vogt plaque method on cultures grown out in the indicated medium in 4 oz prescription bottles(3). The culture fluid was decanted, and the inoculum of virus was added from the same pipette to a series of 12-20 bottles. After incubation for 1 hr at 37°C, bottles of each series were randomly selected and overlaid with calf serum agar overlay, "C.S.", or with amino acid supplement agar overlay "AAS", made as follows: *C.S.*: Agar (2.7 g/60 ml), 33%; calf serum, 2.5%; neutral red (1/1000 of 80% dye), 1.8%; Earle's salt solution 10 x concentrated, 10%; H_2O, 50%; $NaHCO_3$ (58 g/50 ml solution), 2.05%. *AAS*: (solutions as in C.S. overlay) Agar, 33%; basic salt #2 (Table I) 2 x concentrated, 50%; H_2O, 8%; neutral red, 1.8%; amino acid supplement 40 x concentrated, 2.5%; Na_2HPO_4 (5% solution), 0.28%; $NaHCO_3$, 3.8%. Solutions added in order given.

Experimental. The synthetic medium, SM-1, which has been used routinely for outgrowth of monkey kidney cell suspensions, is described in Table I. It consists of a salt solution containing a higher concentration of calcium than is widely used; trace elements; and the organic constituents, cysteine, isoleucine, and d-ribose. The buffering capacity of the medium is increased by the addition of 8×10^{-3} M Tris (hydroxymethyl aminomethane). It may be supplemented with penicillin and streptomycin if desirable. The medium is stable for at least one week at 4°C, and is not used once a precipitate forms on standing. The medium cannot be frozen.

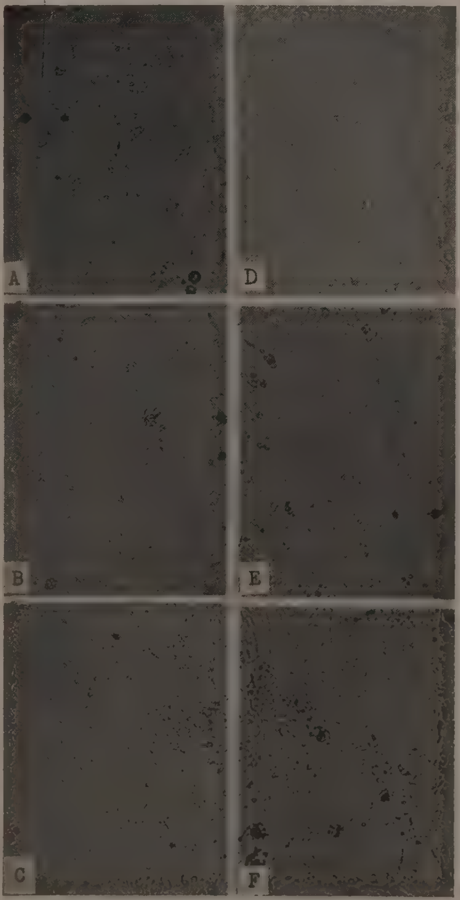

FIG. 1. Outgrowth of monkey kidney cells in SM-1 and comparison with parallel culture in lactalbumin hydrolysate-calf serum "M" medium. A-C = Outgrowth after 2½, 5, and 10 days in "M." D-F = Outgrowth after 2½, 5, and 10 days in SM-1.

The outgrowth of identical suspensions of monkey kidney cells seeded in SM-1 and in the complete medium, M, is shown in the accompanying photographs (Fig. 1). Cells seeded in SM-1 attach more slowly to the glass. The outgrowth in the first 48 hours may be inferior compared to that obtained in the serum-containing medium. By the third day, however, as indicated in the photographs, the outgrowth in SM-1 compares favorably in both amount and quality with the outgrowth in the complete medium. The two sets of cultures are comparable up to the 10th-12th day after seeding. The complete medium must be changed between the 5th and 7th days because of the decline in pH. It is not necessary to change the SM-1 medium before the 8th-10th day.

The choice of medium for replenishment is determined by the studies to be undertaken. Replenishment of cells grown in SM-1 with the M medium results in a further proliferation of the cells after a lag period of 2 days, and survival for a month or more. On the other hand, for studies uncomplicated by the addition of serum, replenishment with an amino acid mixture such as that given in Table II, in the same basic salt solution, gives a useful additional lifetime of 1-3 weeks.

The medium SM-1 was developed on the assumption that freshly isolated kidney tissue, possessing considerable endogenous reserves and biochemical capacities, would require very few organic supplements for outgrowth and survival if placed into a properly adjusted physico-chemical environment. It may be useful to summarize the steps in development. (i) Suspensions of washed monkey kidney cells in Hanks' salt solution were supplemented with single organic nitrogen sources over a range of concentrations and pH. It was found that cysteine was the only supplement in more than 50 tried which gave a significant response; cells suspended in Hanks' solution alone, or in Hanks' solution supplemented with most other nitrogen sources, disintegrated within 24 hours. (ii) The Hanks'-cysteine medium was supplemented with single nitrogen and carbon sources. Several unrelated nitrogen sources increased the extent and duration of outgrowth over that obtained with cysteine alone. On the other hand, d-ribose and several other carbon compounds improved the quality rather than the extent of outgrowth. (iii) The Hanks' cysteine medium, supplemented —rather arbitrarily—with isoleucine and d-ribose, was chosen to investigate the effects of salts and trace elements on outgrowth. The concentration of salts, particularly calcium, was found to be a critical factor for outgrowth in the absence of serum. Both ferric and cupric ions had pronounced beneficial effects. It was also found that when the pH of the medium fell below 7.2-7.3 during the first 4 days, outgrowth was inhibited; cells failed to attach to the glass and the cells already proliferating along the glass became thickened and granulated. Attachment to the glass was also retarded when the medium was more highly fortified with amino acids, phosphates, traces of certain carboxylic acids, or after prolonged metabolism. The morphological changes and lack of attachment have been found to be completely reversible. Cells held in suspensions at 37°C for several weeks, and then adjusted to pH 7.6-7.8 with bicarbonate or supplemented with calcium or zinc, were found to attach and grow into clear monolayers.

TABLE II. Amino Acid Supplement.

	mg/l
l-cysteine • HCl	30
l-isoleucine	40
l-histidine • HCl	10
l-arginine • HCl	7
l-lysine • HCl	8
l-methionine	6
l-threonine	14

Conveniently made from sterile stocks to give 40 × concentrated mixture in H_2O and diluted into salt solution with trace elements and glucose as given in Table I, with 0.9 g $NaHCO_3$ per liter. May be buffered with 8×10^{-3} M Tris at pH 7.6.

The cellular monolayers grown out in SM-1 are so firmly attached to the glass that it has not been possible to remove them by the use of trypsin or versene, or by scraping, without substantial losses. Accurate cell counts have not been obtained. As an estimation of the total number of cells recovered after outgrowth, we have relied on (i) morphological

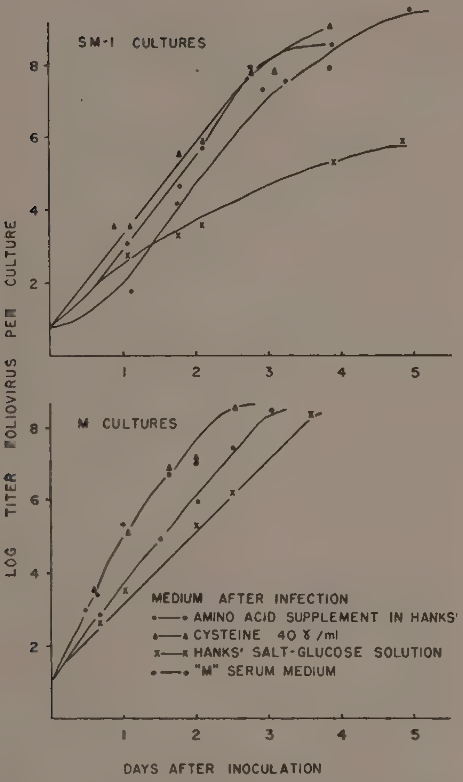

FIG. 2. Production of poliovirus in cultures grown out in SM-1 (top) and in M (bottom); and effect of medium after infection on rate of propagation. A series of 9-day-old cultures grown out in SM-1 and in M in 2 oz prescription bottles, from an inoculum of 1.7 × 10^6 cells/bottle, were infected with 10 plaque forming units of Type 1 poliovirus suspended in the amino acid supplement. Virus adsorbed 30 min. at 37°C. Cultures washed 3 times with PBS and in series of 6 bottles each replenished with 5 ml of medium indicated, supplemented with 8 × 10^{-3} M Tris pH 7.6. Arrow indicates time at which pH was readjusted to 7.4-7.6 with bicarbonate. Virus titered by plaque method, sampling from a different bottle at each point. Last point is pooled sample.

appearance of the culture and (ii) RNA and DNA recovered after a modified Schmidt-Tannhauser fractionation. The RNA and DNA recoveries indicate that after 7-10 days in SM-1, from 0.8-1.5 times the number of cells are recovered as were seeded. This compares with a recovery of 1.0-2.0 obtained in the complete medium changed at the 5th-7th day. The results obtained here for the complete medium agree with those obtained by Youngner(2) on the basis of cell count.

Virus synthesis. As a criterion of the physiological state of the cells after outgrowth in SM-1, the synthesis of poliovirus was studied. The propagation of poliovirus, Type 1, from an initial inoculum of an average of 10 plaque-forming units per culture, was followed in cultures grown out in SM-1 and in cultures of the same suspension grown out in the complete M medium. The propagation of virus throughout the two cultures, and details of the protocol, are shown in Fig. 2 and its legend. It is seen that the total yield of virus obtained from cells grown in the simple medium and inoculated and transferred to the amino acid supplement is the same as that from the control culture grown and infected in the presence of serum. The propagation of virus in the amino acid supplement is somewhat faster than in the M medium, and is more striking when the cells have been previously grown in the serum-containing medium. In this case, there may be as much as 1000 times more virus present in the cultures in the amino acid supplement at the end of 48 hours than in cultures with the complete medium. However, after further incubation of 2 or 3 days, the yield of virus from the M medium approaches that obtained in the amino acid supplement.

Since the amino acid supplement has a higher concentration of cysteine than the M medium, or other media usually used for virus cultivation, the effect of cysteine on virus propagation was studied. The data presented in Fig. 2 show that cysteine can completely replace the amino acid supplement and, even in the case of cells previously grown out in the simple medium, supports a more rapid propagation of virus than the M medium. Preliminary studies(4) indicate that the more rapid propagation of virus is due to a shorter virus growth cycle.

The amino acid supplement may be used advantageously as the nutrient in the agar overlay when assaying virus by the Dulbecco-Vogt plaque technic. The rate of appearance of plaques during assay of poliovirus Types 1, 2, and 3, on M cells overlaid with the

TABLE III. Effect of Amino Acid Supplement in Agar Overlay on Plaque Formation by Polioviruses on Monkey Kidney "M" Cells.

Exp. #*	Virus stock	Overlay medium	Plaques per bottle																														Titer (plaque forming units per ml of stock)
			Day 2										Day 3										Day 5										
1	Type 1, Brunhilde	C. S.†	1	3	2	7	3	2	5	6	3	0	18	9	10	11	7	7	8	6	5		19	13	14	12	13	12	12	10	10	7	62
		AAS†	6	6	7	9	15	12	10	5	5	13	10	9	13	14	21	14	13	10	11	18	12	11	17	16	21	14	13	11	11	18	70
2	Type 2, M.E.F.	C. S.	0	0	0	0	0	0					2	1	1	0	0	3					4	7	2	9	10	8					88
		AAS	7	11	3	5	5	4					8	15	4	11	16	5					10	15	20	14	20	17					220
3	*Idem*	C. S.	0	0	2	0	2	0	1	1	1	0	12	17	16	8	11	16	9	13	5	8	16	23	19	10	17	13	16	9	17		195
		AAS	11	14	13	20	17	14	19	8	12		28	24	17	30	29	30	27	20	23		29	28	20	34	31	32	31	22	29		390
4	"	C. S.	1	1	1	0	0	0	0	0	0	0	2	2	2	1	3	0	0	0	0	0	2	2	4	4	2	0	0	0	0	0	49
		AAS	2	3	2	2	3	3	1	3	2	1	2	6	2	5	5	3	5	3	3	1	23	10	6	10	9	7	9	4	4	6	310
5	Type 3, Saukett (Stock A)	C. S.	4	1	2	4	3	6	2	4	0	0	21	12	11	13	9	14	11	11	12	7	24	17	14	17	17	20	21	19	14	13	88
		AAS	11	16	16	16	13	15	3	11	8	4	23	26	23	22	21	20	13	19	20	13	28	29	26	27	23	27	16	22	21	20	119
6	*Idem*	C. S.	0	0	0	0	0	0	0	0	0	0	10	6	3	4	6	6	4	5	7	10	14	13	9	5	9	11	6	8	12	11	49
		AAS	12	2	3	12	4	0	6	5	5	4	22	13	6	8	14	10	7	9	13	4	23	18	11	13	20	16	13	12	19	8	78
7	Type 3, Saukett (Stock B)	C. S.											0	0	1	1	0	0					2	1	3	3	1						10
		AAS											2	1	5	6	1	6					6	9	11	10	5	7					30

* Exp. 1: .2 ml inoculum/bottle. Exp. 2, 3, 4: Repeat titrations with stock diluted 1.33×, 3×, and 7×, using .1, .2, and .2 ml inoculum/bottle, respectively. Exp. 5, 6: Repeat titrations with Stock A Saukett, .2 ml inoculum/bottle. Exp. 7: .2 ml inoculum/bottle, Stock B Saukett.

† C. S. = Calf serum in agar overlay. AAS = Amino acid supplement in agar overlay.

amino acid supplement in agar compared to the rate of appearance in the calf serum-agar overlay(5) is given by the data in Table III. Each assay was done on outgrowth cultures from kidney cells from different monkeys. The variation in final titer obtained for Types 2 and 3 indicates the variable susceptibility of the different cultures to these types. With all 3 poliovirus types, plaques appeared more rapidly in the amino acid supplement-agar overlay than in the calf serum-agar overlay. This acceleration was particularly striking with the slower growing Types 2 and 3. The final titer on Types 2 and 3 was also higher in every assay in the amino acid supplement than in the calf serum.

The susceptibility of SM-1 cells to poliovirus has been studied by the plaque technic. The data already presented in Table III indicate the rate and number of plaques expected with the different viruses on M cells with the amino acid supplement overlay. A comparative assay on SM-1 cells and M cells with this overlay would indicate any difference in the intrinsic cellular susceptibility to the virus. The SM-1 cells are sensitive to neutral red, and it is necessary to add the neutral red several days after the agar to develop the plaques. Types 1, 2, and 3 were assayed on a series of 10 bottles each of SM-1 cells and of M cells, grown in parallel from the same suspension of trypsinized kidneys. After decanting the growth fluid, the bottles were mixed so that virus inoculum was added from the same pipette in a random order to the series of 20 bottles. Neutral red was added 3 days after the agar overlay, and the plaques were read 4 hrs later. The data in Table IV show that the number of plaques developed on SM-1 cells was the same as the number developed on the M cells. This finding indicates that the cultures grown out in the simple medium have the same intrinsic susceptibility to poliovirus as the usual cultures grown out in serum. It also suggests that the difference in plaque count between M cultures in the calf serum-agar overlay and in the amino acid supplement-agar overlay is a reflection of the metabolism of the cells prevailing in these two media.

TABLE IV. Comparative Susceptibility of SM-1 and M Cultures to Poliovirus.

Virus type	Culture type	Overlay medium	No. of plaques/bottle after 3 days										Avg plaques per bottle
1	SM-1	AAS	12	13	10	11	18	14	11	16	13	27	14.5
Brunhilde	M	AAS	11	10	14	13	14	21	13	11	10	17	13.4
2	SM-1	AAS	9	9	5	11	8	13	8	14	6	7	9.0
M.E.F.	M	AAS	11	10	9	8	8	11	9	13	10	5	9.4
3	SM-1	AAS	10	6	8	4	4	1	8	7	5		5.3
Saukett	M	AAS	9	10	3	9	2	6	2	6	2	5	5.4

Discussion. The present studies indicate that suspensions of monkey kidney cells may be grown out in a simple synthetic medium in the absence of any added protein and compare favorably, in appearance and capacity to support poliovirus synthesis, with suspensions grown in the serum-containing M medium.

Under the conditions which have been studied, cysteine was found to be a specific requirement for successful outgrowth. The function of cysteine is not clear. It has not been possible so far to replace it with inorganic reducing agents, or with other organic sulfur compounds. In view of the effect of slight changes in the ionic balance of the medium during outgrowth, it is possible that these efforts were unsuccessful because the agents interfered with the necessary ionic conditions.

Outgrowth in the absence of serum was found to be sensitive to the concentration of the different salts, particularly calcium. A concentration of calcium as high as 4 times that occurring in normal serum has been incorporated into the medium. Although calcium may regulate many metabolic activities, it is considered that its beneficial effects during outgrowth may be due in part to protection of the cells during the physico-chemical adjustment to *in vitro* conditions. It may easily be observed that monkey kidney cells agglutinate at pH 4.5, suggesting a high concentration of carboxylic acid residues at their surface. If this is the case, at an alkaline pH, calcium may serve to stabilize the cells by shielding of the charged groups. It is also possible that calcium serves as a bridge through which the carboxylic acid groups are bound to the negatively charged glass. If this is a prime mechanism for the anchoring and spreading of cells along the glass surface, the pH, calcium concentration, and anionic components of the medium would be regulating factors for successful outgrowth.

Serum has heretofore been an absolute requirement for the outgrowth of monkey kidney cells. The fact that cells may be grown out and maintained for 2 to 3 weeks in the absence of serum suggests that its role is not predominantly nutritive during this period. It is possible that serum is largely protective, serving as an effective buffer to maintain the particular environment necessary for outgrowth. Biophysical studies have indicated that the elasticity of the mammalian cell membrane is probably due to tightly bound serum protein(6). It should be possible to simulate to some extent the stability afforded by bound protein by the adjustment of the physico-chemical environment. Consideration of some of the physical factors involved in stability and reversible attachment may enable one to design the medium of choice for the particular studies in tissue culture to be undertaken.

One immediate application of these findings would seem to be the production of vaccine from high titer virus obtained from monkey kidney cells grown in a medium free of added animal proteins. The binding of serum protein by animal cells means that after outgrowth in serum, even after washing, there is considerable contamination with serum protein. Although there has been little evidence of protein sensitization after inoculation of vaccines grown in monkey kidney cultures, the problem has raised a considerable controversy, particularly as to the effect of repeated injections over the years.

Summary. A synthetic medium containing only cysteine, iso-leucine, d-ribose in a salt-glucose medium, buffered with Tris (hydroxymethyl aminomethane) supports the outgrowth of monkey kidney cells. The outgrowth is comparable in both rate and amount with the lactalbumin hydrolysate-calf serum (M) medium and supports the synthesis of poliovirus. Propagation of poliovirus throughout a culture in either liquid or agar medium occurs more rapidly in the presence of a simple amino acid supplement than in the serum-containing medium. Cysteine can completely replace the amino acid supplement for propagation of Type 1 in liquid cultures.

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Effects of 2-Ethylamino-1,3,4 Thiadiazole HCl on Uric Acid Production in Man.* (22295)

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(Introduced by Joseph H. Burchenal.)

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A compound having the formula, 2-ethylamino-1, 3, 4 thiadiazole HCl, was found by Oleson, *et al.*(1) to have antitumor activity in various laboratory animals. Further testing by Clarke(2) confirmed the activity against sarcoma 180 and Burchenal and Dagg (3) demonstrated a definite prolongation of the survival time of mice with transplanted leukemias 82 and 8174. Because of its antitumor effect in animals, Farber(4) initiated therapeutic trials in humans. He has supplied us with preliminary clinical dosage data.

Materials and methods. Clinical evaluation by our group was started in patients with far-advanced cancer. This report deals with the observations made in the 8 adult patients treated to date. The thiadiazole was given once daily by mouth, in doses of 1-4 mg/kilo/day. In most cases therapy was continued until the development of oral toxicity. Frequent physical examinations, appropriate roentgenograms, and hematological and biochemical determinations were performed before, during and after treatment. In 5 of the 8 patients herein reported daily serum and urine uric acid determinations were performed. The method employed for the determination of uric acid was that of Archibald as described in detail by Forsham, *et al.*(5). For the urine determinations this was modified as follows: Duplicate specimens were set up. The first specimen was treated in the usual manner. To the second specimen, after dilution 0.01 ml Worthington liquid uricase[†] was added to 5 ml diluted urine in a Coleman, Jr. cuvette and the specimen was incubated at 37°C for 90 minutes. After incubation the procedure was completed as with the first specimen. The color remaining in the second specimen represented non-uric acid chromogens and was subtracted from the total. All

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[†] Worthington Biochemical Co., Freehold, N. J.

TABLE I. Clinical and Uric Acid Data of Patients Treated with 2-Ethylamino-1,3,4 Thiadiazole HCl.

Age	Sex	Diagnosis	Daily dose, mg/kg	Days	Serum U.A. (mg %) Pre Rx	Serum U.A. (mg %) During Rx	Excess urine U.A., mg
25	♂	Melano CA	2	9	—	—	—
			4	3	—	—	—
38	♂	LSA	2	7	6.3	17.6	—
32	♀	Melanoma	2	7	—	—	—
			4	4	—	8.9	—
			6	4			
			4	11	1.9	6.0	—
62	♂	CA tongue	4	6	5.9	19.3	2160
			4	5	6.4	24.6	4270
20	♀	Salivary CA	4	3	3.9	13.8	1272
			4	4	4.5	13.2	1400
40	♀	CA breast	4	7	4.6	15.0	4176
			4*	3	4.8	12.9	2509
			4*	5	4.1	12.1	4951
			4*	6	3.6	11.6	7216
64	♂	CA kidney	4	4	5.9	13.2	4879
39	♀	CA cervix	4	5	6.7	11.2	1469

* Given with equal doses of nicotinamide.

urines were analyzed in this manner. In addition occasional blood specimens were subjected to the uricase test to determine the specificity of those values reported as uric acid. In no instance was any significant amount of non-uric acid chromogen found in the blood.

Results. No evidence of tumor regression so far has been detected in any of the patients treated. Toxic effects have been limited to severe glossitis which occurred regularly in 4-6 days at a dose of 4 mg/kg/day. Smaller doses were tolerated for longer periods of time but similar toxicity occurred when approximately the same total dosage had been achieved. The clinical and dosage data are given in detail in Table I.

An interesting and as yet unexplained biochemical abnormality that occurred regularly was the observation that coincident with administration of the drug there was a prompt and marked elevation of serum uric acid which promptly returned to normal on cessation of administration of the agent. In order to attempt to elucidate this phenomenon, studies of urinary uric acid excretion were done in 5 of the patients. With each course of therapy there was found to be a rise in urinary uric acid excretion occurring promptly and paralleling the rise in serum uric acid. Table I indicates for each course of therapy the excess urine uric acid as compared with the average urine uric acid for a similar control period.

Fig. 1 illustrates the observations made in

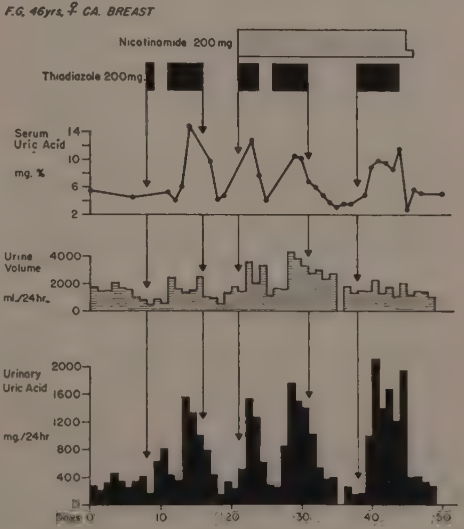

FIG. 1. Serum and urine uric acid in a patient treated with 2-ethylamino-1,3,4 thiadiazole HCl and nicotinamide.

one of the patients to whom this agent was administered. This patient, a 46-year-old woman with carcinoma of the breast and diffuse osseous metastases received 4 separate courses of medication. The dosage was 200 mg daily for 3 to 6 days in each course. On each occasion there was a rise in serum uric acid from normal levels of less than 6 mg% to levels of 10.6 to 15.0 mg%. Simultaneously the urine uric acid rose from control levels averaging 350 mg per day to levels of 1500 to 2300 mg per day. With each course severe glossitis occurred and required cessation of treatment. There was no definite evidence of regression of tumor tissue in this patient during or after treatment. Her blood urea nitrogen remained unchanged throughout this period of observation.

Inasmuch as Oleson(1) demonstrated and Clarke(2) confirmed that nicotinamide was capable of reversing the anti-tumor effect of thiadiazole, it was thought of interest to evaluate the effect of nicotinamide upon the uric acid abnormality produced by thiadiazole. Simultaneous administration of 200 mg daily of each agent produced a rise in serum and urine uric acid which was similar to that produced by thiadiazole alone. Nicotinamide did not prevent the development of oral toxicity.

Discussion. The mechanism of the uric acid abnormality occurring with administration of 2-ethylamino-1, 3, 4 thiadiazole HCl is not understood. The fact that urinary and serum uric acid rose in parallel would indicate that renal mechanisms are not involved. The absence of any indication of tumor regression in these patients renders unlikely but does not completely rule out the possibility that the uric acid was a product of degradation of the nucleic acid of tumor tissue. In the case illustrated an excess of approximately 7200 mg of uric acid above the pre- and post-treatment levels was found in the urine during and immediately after a single course of treatment. Since the average content of nucleic acid in tissue is 1% and since 20% of nucleic acid is composed of purines, calculation of the amount of tissue from which this 7200 mg of uric acid could derive reveals a figure of 3600 g. It might be expected that lysis of this amount of tumor or other tissue would be detectable. A third possibility is that there is a direct increase in uric acid synthesis, occurring as a result of the administration of this compound. This possibility has been neither proven nor disproven. Further studies are under way to investigate this problem and to attempt to correlate the rise in uric acid with nitrogen balance.

Summary. Oral administration of 2-ethylamino-1, 3, 4 thiadiazole HCl to patients with advanced cancer has been shown to cause a marked rise in serum uric acid levels and in total urinary uric acid excretion.

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The Structure of Nuclear Membrane in Larval Gonads of *Heliothis obsoleta*.*†‡ (22296)

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Evidence from electron microscope studies suggests that the nuclear membrane, at least in part, is not a continuous structure but is interrupted at intervals by pores, nodes, granules or ring-like formations. For example, the nuclear membrane of the amphibian egg is a double-layered structure with a continuous inner layer and a porous outer layer(1). The nuclear membrane of *Amoeba proteus* likewise is a 2-layered structure, but here the porous layer constitutes the innermost and the continuous layer the outermost lamella of the membrane(2,3). Other cells showing some form of porous structure in the nuclear membrane are oocytes of the salamander(4), kidney tubule cells(5), acinar cells of the pancreas(6), nerve cells from spinal ganglia (7,8), insect salivary gland cells(9-11), echinoderm eggs(12), several different types of rat cells(13), and chick embryonic cells (14).

Since the evidence for the porous nature of the nuclear membrane is based upon relatively few studies and since, if true, it would have important bearing on our notion of the mode of transfer of materials through it, further studies related to this subject seem warranted.

Materials and methods. Pieces of gonads from the larval insect *Heliothis obsoleta* (*Lepidoptera,* corn ear worm) were fixed for 30 minutes in buffered osmic acid at pH 7.25 (15), embedded in methacrylate(16) and sectioned at 0.025 μ by means of an International Rotary Minot type microtome(17) equipped with a glass knife(18). In Fig. 1, parts (1), (2), (3), and (4) were made by an RCA model EMU-2B electron microscope; part (5) was made with an RCA model EMU-3B electron microscope.

Results. The appearance and distribution of the nuclear membrane pores, or annuli, are clearly displayed in Fig. 1. The pores are best observed in oblique sections where they can be seen to be evenly distributed throughout the nuclear membrane at an average distance apart of approximately 1300 A (Fig. 1-1, 1-2, 1-5). In Fig. 1-2 the section is through a folded membrane showing bits of it (A) cut obliquely and the remaining part cut more or less longitudinally through the long axes of the pores.

The pores have an average diameter of about 280 A. They are encompassed by a relatively dense portion of the nuclear membrane measuring approximately 320 A in thickness (Fig. 1-3 and 1-5). Thus, the total diameter of the pore and surrounding membrane is approximately 920 A. In some preparations the relatively dense layer lining the pores appears smooth (Fig. 1-1, 1-2, and 1-3), while in others a somewhat granular appearance is observed (Fig. 1-5). The small dark granular mass appearing in the center of some of the pores (Fig. 1-5) may possibly represent material that was in the process of diffusing through them at the time the tissue was fixed.

Sections cut through the long axes of the pores show the profiles of the membranes constituting the walls to be darkly outlined (Fig. 1-2 and 1-4). In Fig. 1-4 the nuclear membrane is displayed as a single layer with an estimated thickness of from 60 to 70 A.

Discussion. From the literature cited in this paper it becomes evident that in many types of cells the nuclear membrane contains porous structures. Their orderly arrangement within the membrane and the fact that they have been demonstrated independently

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‡ Thanks are due Dr. T. C. Evans for permission to use the electron microscope for part of this study.

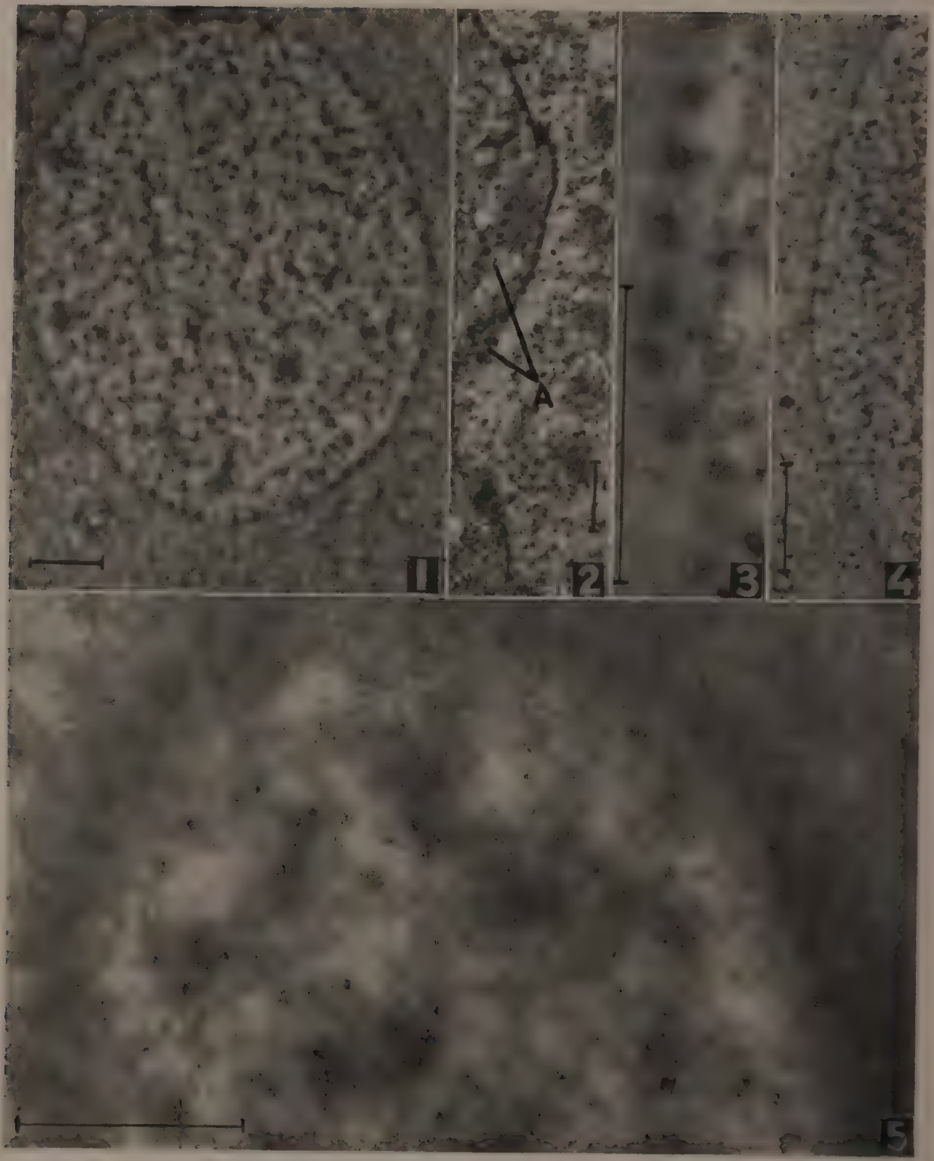

FIG. 1. Section through nuclear membrane showing pores cut either obliquely (1), (2A), (3) and (5), or longitudinally (2) and (4). The scale represents 1 μ.

by several different investigators is evidence against the view that they are artifacts. It has been suggested that they function as passageways for the diffusion of large molecules through the membrane(6).

It has been demonstrated that in certain cells bits of the nuclear membrane may be pinched off into the cytoplasm where they may contribute to the formation of the endoplasmic reticulum(10,11). In other cells, the outer layer of the membrane is thought to be continuous with the endoplasmic reticulum; in such cases, it has been calculated that about one-tenth of the nuclear surface is in direct contact with the cytoplasmic matrix through pores, and the remaining surface is in indirect contact with it through the enclosed cavities of the endoplasmic reticulum (13). Such a close relationship between the nuclear membrane and endoplasmic reticulum is not apparent in the germ cells of the larva *Heliothis obsoleta*.

Summary. 1. Electron microscope studies on thin sections of germ cells of the larva *Heliothis obsoleta* have revealed the presence in the nuclear membrane of pore-like structures which are spaced about 1300 A apart. The pores are approximately 280 A in diameter and are surrounded by a relatively dense wall about 320 A in diameter. Thus, the diameter of the pore plus the surrounding wall is approximately 920 A. 2. The pores appear to be of a size that would permit the passage of large molecules through the nuclear membrane.

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Ineffectiveness of Sulfonylureas in Alloxan Diabetic Rats.* (22297)

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In explanation of the mechanism whereby the sulfonamide derivative, 1-butyl-3-p-aminobenzenesulfonylurea (BZ55) produces hypoglycemia in animal and man, Franke and Fuchs(1), Bertram, Bendfeldt and Otto(2) and Achelis and Hardebeck(3) postulate that the sulfonylurea interferes with the production of glucagon. In support of this hypothesis, these investigators refer to studies by others who demonstrated that some sulfonamide derivatives may reduce the blood sugar of alloxan diabetic animals(4,5). Further, Achelis and Hardebeck state that the sulfonylurea used by Franke and Fuchs(1), and by Bertram, *et al.*(2), produces a decrease in the blood sugar of some alloxan diabetic rabbits though they do state their impression that there may be no decrease in the blood sugar of severely diabetic alloxanized rats. In view of the paucity of data concerning this question, it became pertinent to determine whether or not the sulfonylureas are hypoglycemic in the alloxanized diabetic rat.

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Method. Male rats, weighing from 150 to 250 g were given a subcutaneous injection of 180 mg alloxan per kilogram as a 5% solution. Forty-eight hours thereafter, those animals which developed glycosuria were maintained on 1 unit protamine-zinc insulin[†] per day. Approximately 1 month later, the insulin was discontinued for 2 days, and after an overnight fast, 15 rats which exhibited a significant glycosuria were selected for study. A group of 15 normal male rats weighing from 150 to 250 g were used as controls after an overnight fast. Both groups of rats were given a gavage of 100 mg 1-butyl-3-p-tolylsulfonylurea[‡] per kilogram body weight as a 2% solution in 0.5% sodium bicarbonate, adjusted to pH 8.0. Immediately before and at hourly intervals for 5 hours after the gavage, blood samples were taken from the clipped tails into 0.1 ml pipettes. The blood sugar concentration was determined by the Nelson procedure(6). The blood sugar concentration for each interval after the gavage was expressed as the per cent of the pregavage level for each animal.

Results. The mean ± SE response of the blood sugar concentration to the ingestion of tolylsulfonylurea by the normal and alloxan diabetic groups of rats is illustrated in Fig. 1. The mean ± SE initial blood sugar concentration was 83.0 ± 3.0 mg in the normal group and 352 ± 17.5 mg% in the alloxan diabetic group. Whereas the normal rats developed a statistically highly significant diminution in the blood sugar level within 1 hour after the ingestion of the sulfonylurea which persisted for four hours, no significant change in the blood sugar occurred in the diabetic rats.

Discussion. The administration of tolylsulfonylurea to fasted alloxan diabetic rats does not produce any significant change in the blood sugar whereas normal rats exhibit a marked hypoglycemia under similar conditions. Since the administration of alloxan results in necrosis of the beta cells of the Islets of Langerhans and a consequent cessation of insulin production, the hypoglycemic response to the ingestion of tolylsulfonylurea must be related to the availability of insulin. Further, the absence of any hypoglycemic response in the diabetic rats suggests that the hypoglycemic response of the normal rat cannot be due to an inhibition of glucagon production or utilization. Likewise, the hypoglycemic response of the intact animal cannot be due to some acute non-specific hepatotoxic action of the sulfonylurea since such action would be present in the alloxanized as well as the normal animal.

The above indicates that the response of normal animals is due to an increase in the availability of insulin. Such an increase may result from either an increased production of insulin consequent to direct stimulation of the Islets of Langerhans or to a decreased destruction of insulin consequent to an inhibition of the enzyme which catalyzes the destruction of insulin (insulinase), or to both factors. That the inhibition of insulinase plays a role in the response to the sulfonylurea by normal rats is revealed in the observation that the tolylsulfonylurea is a noncompetitive inhibitor of insulinase *in vitro* and *in vivo*(7).

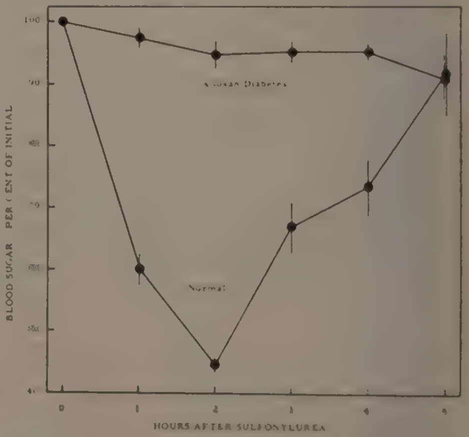

FIG. 1. Effect of tolylsulfonylurea on blood sugar of normal and alloxan diabetic rats. Blood sugar concentration at each hourly interval is expressed as % (mean ± SE) of initial concentration.

[†] We are indebted to the Eli Lilly Co. for generous supplies of insulin.

[‡] We are indebted to Dr. C. J. O'Donovan of the Upjohn Co. for generous supplies of 1-3-butyl-p-tolylsulfonylurea ("Orinase").

Summary. Oral administration of 100 mg 1-3-butyl-p-tolylsulfonylurea per kg body weight to normal rats results in a marked hypoglycemia within one hour which persists for 4 hours. No significant change occurs in blood sugar of similarly treated alloxan diabetic rats.

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Effect of CO_2 on Blood Lactic Acid in Cats.* (22298)

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Fenn and Asano(1) have observed in cats an increased acidity or fall in the alkali reserve of the blood during CO_2 inhalation, which could not be explained by movements of sodium, potassium, or chloride between tissues and plasma, and Shaw and Messer(2) have similarly observed a fall in the bicarbonate level during CO_2 breathing.

These experiments were undertaken to help clarify these findings and to identify if possible the acid mobilized by CO_2.

Procedure. The effect of high CO_2 tension on blood lactic acid was investigated in cats. Each animal was anesthetized by intraperitoneal injection of dial with urethane. Urethane has been found to raise the blood sugar, but not the lactic acid and allows for a large and rapid effect of adrenalin on the blood lactic acid(3). Four different gas mixtures were used, 10%, 20%, 30%, and 40% CO_2 in oxygen. Thus in every case the experimental animal was assured of a sufficient supply of oxygen to avoid any possibility of anoxic lactic acid formation. The desired gas mixture was inhaled by the cat from a spirometer through respiratory valves and a tracheal cannula. Blood samples were taken from the right carotid artery and were immediately deproteinized with trichloroacetic acid to stop glycolysis. After the tracheal and arterial cannulation, heparin was injected (10 mg/kg of body weight). One sample was always taken just before the onset of CO_2 inhalation. The lactic acid was determined colorimetrically by the method described by Barker and Summerson(4).

Results. The findings of these experiments are illustrated in the accompanying graphs. Increments of lactic acid in m. eq./liter above the rather variable initial blood level are plotted against time after the beginning of CO_2 inhalation. To save space, a logarithmic time scale has been used. In the graphs on the left, the different concentrations of CO_2 were inhaled for 10 minutes, (solid lines) after which the cat breathed air, (dotted lines) while in those of the right, the CO_2 was inhaled for one hour or more and a few curves are continued as dotted lines during recovery breathing air. The initial levels of lactic acid corresponding to each curve are indicated by points with appropriate symbols plotted just to the right of each graph.

With 10% CO_2 there is often a slight fall, but never a definite increase in lactic acid. The increase with 30% and 40% CO_2 is on the average somewhat greater than with 20%. The cats were unable to take 40% CO_2 for

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* This work was supported in part by the Ernest L. Woodward Fund of the University of Rochester.

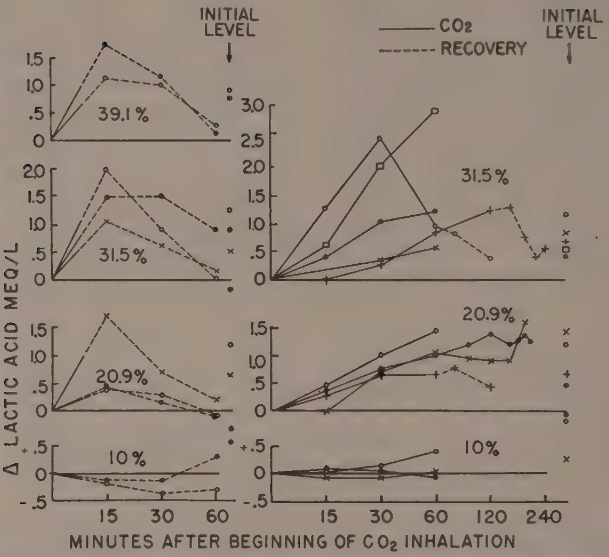

FIG. 1. Changes in lactic acid concentration of blood resulting from inhalation of different concentrations of CO_2, as indicated on the graph. Abscissae represent time after beginning of CO_2 inhalation in logarithmic units. Duration of CO_2 period was uniformly 10 min. in the graphs on the left; in graphs on the right the duration is longer as indicated by length of solid line. Broken lines represent recovery periods breathing air. The initial level of lactic acid before CO_2 is indicated by the appropriate symbol for each experiment on the right of the graph.

periods much longer than 10 minutes without cessation or serious depression of respiration and the possibility of anoxia. With 10 minute exposures to CO_2 the lactic acid reached a maximum at 15 minutes and then returned to normal in about one hour. With longer exposures the lactic acid continued to rise as long as the CO_2 was breathed or at least for one hour. In 3 out of 4 cases where recovery was followed after a prolonged CO_2 exposure, there was a slight additional rise of lactic acid when the CO_2 was discontinued. A similar "off effect" may account for the fact that the 15 minute values in short term exposures (taken after 5 minutes of recovery) seem to be on the average higher than the 15 minute values during the prolonged breathing of CO_2. No correlation was found between the initial level of lactic acid and the magnitude of the increase under similar CO_2 concentrations and no other single factor seems to account for the wide variation in response that occurs in different cats with identical CO_2 mixtures. It is likely that a combination of experimental factors and individual differences is involved.

Discussion. The release of adrenalin by CO_2 inhalation in cats has been observed by the resulting contraction of a previously denervated nictitating membrane by Fenn and Asano(1). It is assumed that the rise in blood lactate in these experiments is at least partially due to the action of the liberated adrenalin(5).

The rise in lactic acid found in these experiments after inhalation of 30% CO_2 for 60 minutes was 0.5 to 2.9 m. eq. per liter. Fenn and Asano(1) under comparable conditions found a decrease in bicarbonate (measured at constant pCO_2) of 4.16 m. eq. per liter. The lactic acid therefore is only partially sufficient to explain the acidity previously observed. In the experiments of Shaw and Messer(2) it was reported that inhalation of 10-11% CO_2 for 40 minutes caused a decrease of bicarbonate (at constant pCO_2) of 1.9 m. eq. per liter (4.2 vol %). This figure is in good agreement with the results of the present experiments although there seems to be some discrepancy in the threshold CO_2 concentration required for lactic acid formation. In the experiments reported here 10% CO_2 never caused a rise in lactic acid concentration, but this is close to the threshold and a deeper anesthesia or a slightly larger respiratory dead space in the experiments of Shaw and Messer might have increased the alveolar CO_2 sufficiently to cause an effect.

In contrast to the results of these experiments, high CO_2 tensions have been reported to cause a fall in lactate in dogs(6), as well as an increased breakdown of phosphocreatine in isolated muscle(7). Further an increase in lactate has been reported during a respiratory alkalosis in dogs(6) and in a heart-lung preparation(8), and also following the injection of an alkaline solution in dogs(9,10). This suggests a metabolic buffering mechanism whereby more lactic acid is produced when the pH rises, and less lactic acid when the pH falls. In consideration of such data, the results of the present experiments, showing an increase in lactic acid on inhalation of CO_2, require some explanation, possibly on the basis of a species difference. The data of Giebisch, Berger, and Pitts(6) indicate a fall of approximately 1.0 mMol of lactate per liter in dogs that had been breathing 20% CO_2-80% O_2 for 75 minutes, while in the experiments on cats presented in this paper, there is never any evidence of a drop in lactate at any time up to nearly 3 hours with 20%, 30%, or 40% CO_2 in the inspired air.

This apparent contradiction in the results with cats and dogs may be explainable by a greater dominance or influence of the sympathetic nerves in cats(11). It is possible that the metabolic buffering mechanism seen in dogs tends to occur also in cats during CO_2 inhalation, but is masked by a release of adrenalin, which enhances the breakdown of muscle glycogen to lactic acid. If there is any purposefulness to be discerned in the reaction observed in cats, it must depend upon some features of the response other than the change in pH since lactic acid enhances the acidity caused by CO_2. Guinea pigs apparently behave like cats in this respect since Schaefer, King, Mego, and Williams(12) have reported an increase of 5.3 m. eq./liter of lactic acid 1 hour after breathing 30% CO_2 in O_2.

Summary. Inhalation of 20-30% CO_2 in oxygen by cats causes a progressive increase in the lactic acid concentration of the blood, due in part to the liberation of adrenalin.

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Improved Method for Determination of Inorganic Sulfate in Biologic Fluids.* (22299)

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(Introduced by P. K. Bondy.)
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Numerous adequate technics for the determination of fairly large amounts of inorganic sulfate in biologic material have been available for many years(1-3). However, the low concentration of inorganic sulfate in the plasma (2-3 mg % as $SO_4^=$) has rendered most of these methods inadequate for use with relatively small amounts of plasma (*i.e.* one to 2 cc). The basic technic which has appeared most applicable to the analysis of small quantities of inorganic sulfate in biologic fluids is benzidine precipitation of the sulfate(4). Benzidine, a weak organic base, forms stable salts with strong mineral acids. The sulfate is insoluble in the presence of excess benzidine in acid solution and particularly in the presence of organic solvents such as acetone and alcohol(4). The optimum conditions necessary for the precipitation of benzidine sulfate have been extensively studied(4-6). The benzidine sulfate must be quantitatively determined either titrametrically(7) or colorimetrically(8). In the former, the benzidine sulfate is titrated with NaOH by the following reaction: $C_{12}H_8(NH_2)_2\,H_2SO_4 + 2NaOH = C_{12}H_8(NH_2)_2 + Na_2SO_4 + 2H_2O$. According to Peters (4) the free benzidine is so weak a base that it is not alkaline to phenolphthalin or phenolred. The H_2SO_4 combined with it may be titrated as the free acid. This procedure, although accurate, requires titrating at 100°C. Letonoff and Reinhold(9) made a notable advance in the colorimetric determination of benzidine by using sodium β naphthoquinone-4-sulfonate as a color reagent. With benzidine in alkaline solution this compound develops a red brown color which changes to red on addition of acetone. However, the spectral characteristics and stability of the final color were not adequately studied. The precipitate of benzidine sulfate, once formed, must be separated and washed free of excess benzidine. As emphasized recently by Dodson and Spencer(6), considerable losses of the precipitate may occur during these steps. There is a tendency for the precipitate to break away during decantation regardless of the type of centrifuge tube used; this they prevented by adding small amounts of powdered glass as a rough surface on which the precipitate could be packed down. The benzidine sulfate has usually been precipitated from an acetone or ethanol solution and subsequently washed with either of these solvents. In the present study it was found that small but consistent losses of the benzidine sulfate precipitate occurred when washed with either acetone or ethanol. This could be prevented by washing with a mixture of ⅔ ethanol and ⅓ ethyl ether. Benzidine sulfate is considerably more insoluble in ethyl ether than in either acetone or alcohol.

By utilizing powdered glass(6), washing the precipitate with an ethanol-ether mixture and employing the color reagent, sodium β naphthoquinone-4-sulfonate(9) an improved technic for the measurement of small amounts of inorganic sulfate (10 or more μg) was obtained.

Materials and methods. Reagents. 1. H_2O, redistilled; 2. Ethanol, 95%, redistilled over NaOH pellets; 3. Benzidine, (Fisher Scientific Co., B-259), 1 g in 100 ml redistilled ethanol, preserved in dark bottle; 4. Trichloroacetic acid, 20%, redistilled with 208 mg $BaCL_2$ in 2 ml H_2O/200 g trichloroacetic acid (analytical reagent); 5. Sodium borate, (Fisher Scientific Co. S-248), 1 g in 100 ml of 0.1 *N* NaOH, stored in pyrex bottle. 6. Sodium sulfate, anhydrous, A. R., 1.4788 g/l of water to give a 100 mg % $SO_4^=$ solution. 7. Benzidine hydrochloride, purified according to Letonoff and Reinhold(9), 0.1606 g benzidine hydrochloride are placed in 200 ml volumetric flask, dissolved in about 50 ml water at 50°C, cooled to room temperature, diluted to mark and stored in the cold; 8. Sodium 1, 2-naphthoquinone-4-sulfonate (S. N.S.) (Eastman-Kodak 1372), purified: 2 g of crude compound are dissolved in minimum amount of boiling water, filtered through heated funnel, salted out with absolute ethanol, by adding ethanol drop wise to filtrate, filtered by suction on Büchner funnel, washed with absolute ethanol, dried with ether and stored in dark bottle. The solution must be freshly prepared each time by dissolving 150 mg in 100 ml water;† 9. Ether, A.R.; 10. Acetone, A.R.; 11. Powdered glass, washed, (Fisher Scientific Co. S-248) 15 ml heavy wall centrifuge tubes are used. All glassware should be rinsed with dilute nitric acid.

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Procedure. All urine and serum samples are diluted to give a concentration of 20-140 μg $SO_4^=$ per cc. To 2 ml of the diluted sample are added 2 ml of water and 4 ml of 20% trichloroacetic acid. Samples are mixed, allowed to stand for 10 minutes and centrifuged for 10 minutes. From the supernatant fluid 2 ml aliquots are withdrawn and introduced into centrifuge tubes. To each of these tubes 5 ml of 1% benzidine solution is added. The mixtures are stirred and stirring rods washed with small amounts of ethanol. Centrifuge tubes are covered with parafilm and placed in ice-bath overnight. On following day‡ 3 to 5 mg of glass powder is added to each tube and precipitated benzidine sulfate is separated by centrifugation at 2250 rpm for 15 minutes. The supernatant fluid is decanted, with care taken not to loosen any of the precipitate. The tubes are drained, inverted on filter paper, 5 to 10 minutes and mouths of tubes are then wiped with tissue paper. The outsides of tubes are washed with acetone by medicine dropper. After draining for 1 minute, the insides of the inverted tubes are rinsed with ethanol and tubes are drained for 5 minutes. The mouths of tubes are again wiped with tissue paper and tubes are placed upright. Ten ml of an alcohol-ether mixture (1:2) is added to each tube and the precipitate suspended by a stirring rod. Care is taken to break the precipitate completely in order to dissolve all excess benzidine. The glass rods are washed with the same alcohol-ether mixture and tubes are centrifuged at moderately high speeds for 15 minutes. The supernatant fluid is decanted and tubes drained for 5 minutes. The tubes are then wiped and washed inside and outside as before. One ml of sodium borate solution is then added, the precipitate is resuspended and the tubes placed into a water-bath with occasionally stirring at about 60°C until the precipitate completely dissolves. To each tube 10 ml of water and 1 ml of the color reagent (sodium 1:2 naphthoquinone-4-sulfonate) are added and allowed to stand for 5 to 6 minutes. Two ml of acetone are then added to each tube and the solutions are mixed, centrifuged and supernatant fluid is poured into colorimeter tubes. Care is exercised that identical numbers of minutes elapse between addition of color reagent and of the acetone to each tube. The optical density of the solutions is determined in the Coleman Junior Colorimeter at 490 mμ. All analyses were done in triplicate or quadruplicate.

Standards. Stock standard benzidine hydrochloride is diluted 30 fold. To 7 centrifuge tubes 1 to 7 ml of this diluted standard solution is added and the volumes made up to 10 ml with water. A blank tube containing 10 ml of water is prepared with each determination. To all tubes sodium borate, color reagent and acetone are added as outlined before.

Results. Characteristics of benzidine-sodium β naphthoquinone-4-sulfonate (S.N.S.) complex. Fig. 1 illustrates the absorption spectrum of final color compound. Maximum optical density occurs at 490 mμ. It is

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† In the present study, in contrast to Letonoff(9), a definite fall in optical density of standards was noted if fresh reagent was not prepared with each set of analyses. However, as the optical density of the standards and unknowns are lowered by proportional amount no error is introduced by using S.N.S. previously prepared and stored in the cold (4°C).

‡ Complete precipitation will occur within 3 hours; leaving samples overnight is a matter of convenience.

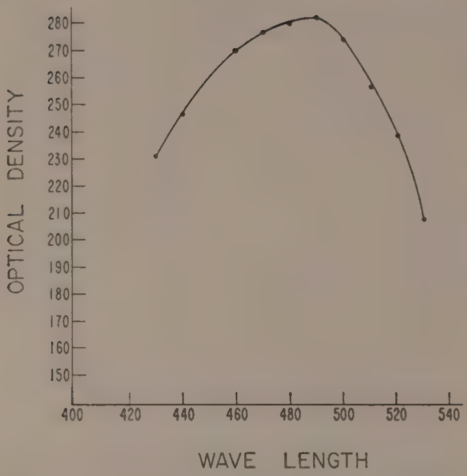

FIG. 1. Absorption spectrum for final benzidine-β naphthoquinone-4-sulfonate compound as determined on Coleman Junior Spectrophotometer.

of interest that this wave length also represents the optimum length for the color developed by interaction of this reagent and amino acids(10), the amine groups of benzidine combining with the S.N.S. to give the observed color. At this wave length, the standard curve follows Beer's law quite closely between zero and 50 μg of sulfate (the benzidine hydrochloride equivalent to these amounts of $SO_4^{=}$). Rarely at 50 μg, and invariably above this level the curve deviates from a straight line as illustrated in Fig. 2. Therefore for maximum accuracy the precipitate of benzidine sulfate should be equivalent to 50 γ of sulfate or less. Although the slope of this curve is readily reproducible small variations in optical density of the standards from one analysis to the next necessitates that a set of standards be run simultaneously with the unknowns. Maximum color development occurs in 5 to 6 minutes and this is stable for at least 2 hours if room temperature is relatively constant. After 2 hours optical density decreases slowly and proportionally in all samples.

Recovery of benzidine sulfate from standard solutions, urine and serum. In Table I are listed recoveries of sulfate from solutions of known concentration. The mean recovery for this series was 98.8% (range 94-104) however, recoveries below 97% were seen only with the sample containing 10 γ of sulfate. Sulfate added to serum and urine was quantitatively recovered (Table II). The mean recovery from serum was 100.5% (range 97-102) and from urine 99.0% (range 97-104). Duplicate determinations on fresh

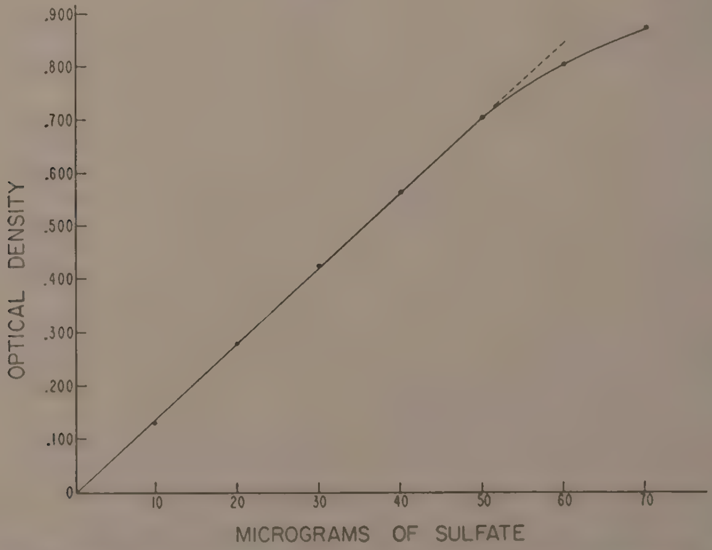

FIG. 2. Calibration curve for benzidine-β naphthoquinone-4-sulfonate compound at 490 mμ.

TABLE I. Analysis of Pure Sulfate Solutions. Five determinations* in each solution.

Sulfate,† μg	Found: Mean	Found: Range	% variation
10	10.1	9.4-10.4	94-104
20	19.7	19.4-20.0	97-100
30	30.1	29.0-30.4	98-103
40	39.7	39.0-40.0	99-100
50	49.9	49.2-52.0	98-104
60	59.5	58.0-62.0	97-103
70	69.5	68.0-70.7	97-101

* Each determination done in triplicate.
† Sulfate present in initial aliquot of solution.

or frozen serum and urine were reproducible within a range of ± 2.5%. If, however, serum or urine were stored at 4-5°C in the unfrozen state for 24 hours or longer an increase in concentration of inorganic sulfate was almost always noted, probably as a consequence of some degeneration of organic sulfates in these biologic fluids.

The mean concentration of inorganic sulfate in the sera of 15 normal subjects was 2.84 mg % (range 2.23-3.90). These values are comparable to those obtained by other investigators using benzidine or other technic (1,3,11).

It has been reported that inorganic phosphates in concentrations found in human urine are coprecipitated as benzidine salts and must be removed prior to precipitating inorganic sulfate(5,7). With the present technic, however, urinary inorganic phosphates in concentrations up to 150 mg % did not interfere with sulfate precipitation. This was investigated by determining urinary sulfates before and after precipitation of phosphate as magnesium ammonium phosphate(12).§

TABLE II. Recovery of Sulfate Added to Serum and Urine.

Serum	Added sulfate, μg	Total: Expected, μg	Total: Found, μg	% of expected $SO_4^=$ recovered
μ				
1 (15.4)*	15.0	30.4	31.0	102
"	30.0	45.4	46.4	102
"	30.0	45.4	44.2	97
2 (30.0)	15.0	45.0	46.0	102
"	15.0	45.0	44.0	98
"	30.0	60.0	60.2	100
3 (41.6)	3.3	44.9	45.0	101
"	6.6	48.2	49.4	102
Urine				
1 (11.2)*	30.0	41.2	39.8	97
2 (22.5)	20.0	42.5	41.5	98
"	15.0	37.5	37.3	100
3 (21.6)	22.5	44.1	44.8	102
"	15.0	36.6	38.0	104
4 (16.0)	22.5	38.5	38.0	99
5 (32.0)	22.5	54.5	55.7	102
"	15.0	47.0	47.6	101
6 (9.6)	7.5	17.1	16.7	98

* Sulfate present in initial aliquot of serum and urine.

The reproducibility and accuracy of the present method was equal to or better than other procedures utilizing larger quantities of plasma or serum(1,2,3,7,13). However, it must be stressed that, *like* all procedures requiring quantitative recovery of a small precipitate, utmost care is essential.

Summary. 1) An improved method for the determination of inorganic sulfate in biologic fluid has been presented.

2) The results compare favorably with other methods requiring larger quantities of serum or plasma.

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§ Powdered calcium hydroxide(4) was found unsuitable as an agent to precipitate phosphate because varying amounts of sulfate were found to be coprecipitated with calcium phosphate.

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Comparison of Pharmacological Effects of Lysine and Arginine Vasopressins.* (22300)

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The neurohypophyseal octapeptides, oxytocin and vasopressin, have been separated from the posterior lobes of oxen as highly purified substances by du Vigneaud and his collaborators(1,2). Related studies of hog pituitary glands led to the isolation of an oxytocin identical in all respects with the oxytocin of the ox(1). Hog vasopressin, however, differs from ox vasopressin in one respect: the arginine of ox vasopressin is replaced by lysine(3). The latter hormones are therefore conveniently designated as arginine or lysine vasopressin according to the amino acid characteristic of each. The pharmacological effects of the two vasopressins differ in one interesting respect which is described in this report.

Material and methods. Two preparations containing lysine vasopressin of different degrees of purity were used: one, a natural lysine vasopressin, purified but not regarded as the pure hormone (No. 686) and the other, an extract of acetone-desiccated hog posterior lobes (No. 700). The purified natural lysine vasopressin was prepared in the Department of Biochemistry of Cornell University Medical College; we are indebted to Prof. V. du Vigneaud for the gift of this preparation. The extract of hog posterior lobe powder was made by suspending the powder in dilute acetic acid at pH 3.0, boiling for 1 minute and filtering after cooling. The solutions of this extract and of the purified natural lysine vasopressin, also dissolved in aqueous acetic acid solution at pH 3.0, were sealed in ampoules and immersed in boiling water for 10 minutes before final storage in the refrigerator at 1.0°C. The vasopressor potency of the stored solutions in terms of U.S.P. standard were as follows: No. 686, 1.34 units per ml, and No. 700, 3.54 units per ml. The absolute potency of the samples at the time of use were 134 units per mg for No. 686, and 1.35 units extracted from 1 mg of powder for No. 700. The methods of assay have already been described(4). They employ intravenous injections in the anesthetized rat (vasopressor response) and lactating rabbit (milk-ejecting response) and in the trained unanesthetized hydrated dog (antidiuretic response). The antidiuretic response was also determined after subcutaneous injection in the hydrated unanesthetized rat by the method of Burn (5). Lastly, the avian depressor response to intravenous injection was measured in the anesthetized fowl by Coon's method(6).

Results. Because the vasopressor assay was considered the most convenient and accurate to perform in terms of expenditure of time, the potency as found by this method was chosen as the standard of comparison with the other pharmacological properties. The pattern of the pressor response to lysine vasopressin with regard to dose-response relationship and duration of action was identical with that elicited by the U.S.P. reference standard or by arginine vasopressin. The variability in potency from ampoule to ampoule is indicated by the following result: 134.3 units per mg with a standard error of ± 5.6 units for No. 686 from vasopressor assays in 12 rats with as many ampoules.

In Table I, the relative potencies of natural arginine and lysine vasopressins compared by the different tests are listed. In addition, the potencies of the extract of hog posterior pituitary powder are recorded; this extract also contained oxytocin which accounts for the high potency of this extract as tested for its effects on the blood pressure of the fowl and

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[†] Research Assistant supported by a research grant (H-1788) from National Heart Institute of the National Institutes of Health, Public Health Service.

TABLE I. Relative Potency of Arginine and Lysine Vasopressins.

	Tests of potency				
Preparation	Pressor† (rat, i.v.)	Antidiuretic (Dog, i.v.)	Antidiuretic (Rat, s.c.)	Avian depressor (fowl, i.v.)	Milk-ejecting (rabbit, i.v.)
Arginine vasopressin (natural)	100	100	100	14	17
Lysine vasopressin (natural)	100	15	110	16	22
Extract of hog post. pituitary*	100	17	88	127	130

* Contained both vasopressin and oxytocin.

† In these experiments, the pressor effects of the 3 preparations in the rat were arbitrarily assigned a value of 100.

on the pressure in the lactating mammary gland of the rabbit. The avian vasodepressor and milk-ejection effects of lysine vasopressin are qualitatively indistinguishable from those of arginine vasopressin. Also the avian depressor-pressor and milk-ejection-pressor ratios show within the limits of experimental error a satisfactory agreement. In contrast to the findings in the above mentioned bioassays, the relative antidiuretic potency of lysine vasopressin after intravenous injection in the dog is much lower than that of its arginine congener and requires more detailed consideration.

Whenever highly purified lysine vasopressin is administered intravenously to the unanesthetized hydrated dog, the antidiuretic response differs qualitatively as well as quantitatively from that observed following the injection of arginine vasopressin. First, the urine flow in the first 5-minute period following the intravenous injection of lysine vasopressin is about the same as in the second 5-minute period, whereas the antidiuresis produced by the arginine compound is commonly much more marked in the second period (Fig. 1). Secondly, lysine vasopressin as assayed intravenously for its antidiuretic action in the dog, has about one-sixth of the potency of arginine vasopressin when both are compared with their vasopressor potencies in the rat (Table I).

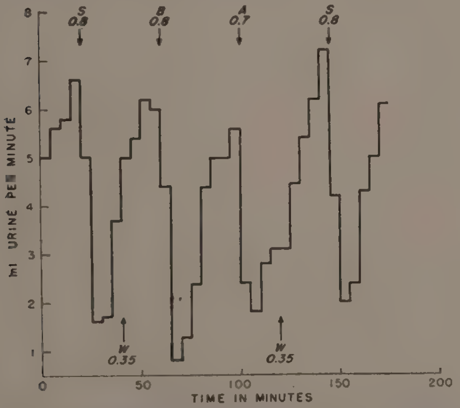

FIG. 1. Antidiuretic response in trained unanesthetized dog after intrav. injections of: S. U.S.P. Posterior Pituitary Reference Standard, 1 mU/ml. B. Extract of human infundibular stem, No. 722 (containing arginine vasopressin), 1:20. A. Purified lysine vasopressin, No. 686, 6.7 mU/ml according to vasopressor assay in the rat. W represents fractions of liters of water by stomach tube in addition to 0.4 liter at –75 and –60 min.

In an endeavor to compare the antidiuretic potencies of the vasopressins by a method employing a different route of administration, assays were performed in the rat by the method of Burn. Eleven groups of 4 animals each were employed using 2 dose levels of each preparation in at least 10 groups (40 rats). The parameter of time to 50% excretion of the water load was chosen since it appeared to be less influenced by the retention of urine in the bladder. Under these conditions, lysine vasopressin, like its arginine relative, evokes an antidiuresis which is equivalent to its pressor potency. To learn whether there is a general difference in the response of the dog and rat to lysine vasopressin, quantitative estimates of the vasopressor potency of the hormone after intravenous injection were made in the dog. The vasopressor action of lysine vasopressin was identical in the dog and rat in terms of U.S.P. standard.

Discussion. Experiments with highly purified arginine vasopressin demonstrated that the antidiuretic hormone is probably identical with the vasopressor hormone of the neurohypophysis of oxen. In the quantitative determination of arginine vasopressin in an extract either type of assay could be employed. With the isolation of another naturally-occurring vasopressin which has a 1:6 murine vasopressor-canine antidiuretic ratio of doses, assays for antidiuretic hormone must take into account the source of the preparation, the species used for assay and the route of administration. The low potency of lysine vasopressin as an antidiuretic agent in the dog could be attributed to a difference in the response of another species. This explanation is untenable as a general statement because the vasopressor potency of lysine vasopressin, in comparison with the U.S.P. standard, is the same in the rat and dog.

Other investigations, some of which are still in progress, indicate that extracts of the neurohypophysis of man, the monkey, dog, rat, ox, sheep and camel, when assayed by intravenous methods, show a 1:1 ratio for vasopressor and antidiuretic potencies. The work described in this paper suggests that arginine vasopressin is the principal if not the only neurohypophyseal hormone responsible for these actions in the above mentioned species.

Summary. 1. The pharmacological effects of lysine and arginine vasopressins are the same with respect to their relative vasopressor, milk-ejecting and avian depressor potencies. 2. The ratio of pressor potency to antidiuretic potency is 1 for arginine vasopressin and 6 for lysine vasopressin when the antidiuretic potency is determined by intravenous injection in the hydrated, unanesthetized dog. On the other hand, this ratio of potencies is 1 for both vasopressins when the antidiuretic potency is determined by subcutaneous injection in the hydrated, unanesthetized rat. 3. Lysine vasopressin, compared with the U.S.P. standard or arginine vasopressin, is equally potent as an intravenous pressor agent in the dog and rat. Potency refers to *relative* potency; potency in units per mg is much higher for purified arginine vasopressin. 4. Lysine vasopressin is peculiar to the hog. The vasopressin of man, the macaque monkey, dog, rat, ox, sheep and camel appears, by pharmacological criteria, to be arginine vasopressin. These conclusions are tentative and must be confirmed by the isolation of the hormone from each species, other than the ox and hog, together with the demonstration of the amino acid composition of each vasopressin.

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Arterial Pressures in Tourniquet Shock. (22301)

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In this study arterial pressure was recorded during the entire period of tourniquet shock in the rat. The object was to correlate the pressure with the course and fatal outcome of the shock state.

Method. Our standard method for the production of shock in rats involves a high unilateral tourniquet applied to the left hind extremity for 5 hours. The technic was described previously(1). White male rats weighing about 250 g were used. Prior to removal of the tourniquet, the animals were anesthetized with 2% Nembutal, given intraperitoneally, and each received 0.2 cc liquid heparin by vein. The right femoral artery was then cannulated (the carotid artery was used in a few instances) and connected to a mercury manometer. Practically all parts of the apparatus were treated with Monocote (Armour) to provide a non-wettable surface. Control readings were taken for several minutes before the tourniquet was released. Direct arterial pressures were obtained throughout the course of shock in 15 animals. In an occasional rat there was need for an extra dose of anesthesia during the period of observation.

Results. The arterial pressure curves were basically similar in all 15 rats with tourniquet shock. Fig. 1 illustrates 3 such curves in animals which died at 1½, 3, and 5 hours respectively after removal of the tourniquet. 1½ and 5 hour animals represented the shortest and longest survivals in the whole group. The mean survival time was 2 hours and 40 minutes.

The initial pressure in the various animals ranged from 104 to 130 mm, and there was no significant change during the control period. Almost immediately after tourniquet release, *i.e.* within a minute or so, there was an abrupt fall in pressure to levels of about 70 to 80 mm. This was probably due to reactive hyperemia in the injured limb as indicated by a bright pink flushing of the paw promptly after the tourniquet was removed. However, participation of nervous factors or possibly toxic material from the damaged extremity is difficult to exclude with certainty.

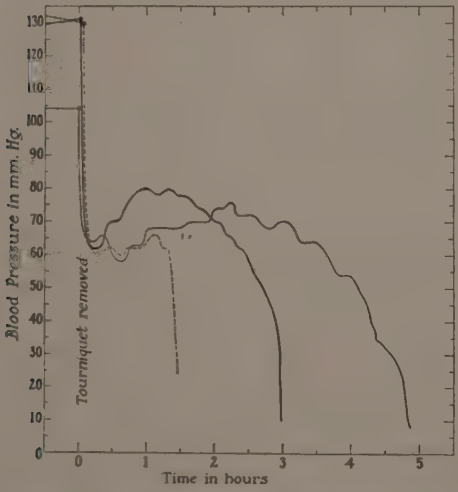

FIG. 1. Arterial pressures in tourniquet shock.

After the initial sharp drop, the arterial pressure showed a further mild decline for a short interval of about 10 to 15 minutes. Then over approximately the next one to 4 hours, depending on the duration of life, and continuing until shortly before death, there was sustained hypotension. During this time, which comprised most of the shock state, the pressure was maintained within a relatively narrow range of about 60 to 70 mm or 70 to 80 mm. That this occurred in spite of progressive shock with marked fluid loss in the injured limb amounting to about 5% of body weight(1) and reduction in effective circulating blood volume to about half the normal value(2), was presumably due to compensatory adjustments of the circulation such as vasoconstriction, increased cardiac action, etc. In fact the compensatory mechanism was probably responsible for the tendency of arterial pressure to rise slightly in some animals during the latter part of the shock state.

Terminally, over a period of a few to about 20 minutes, there was a collapse of the arterial pressure which was associated with death. This was the only interval during the entire course of shock when the pressure fell below 40 mm Hg. The most abrupt decline occurred in animals with the shortest survival time (Fig. 1).

The reason for the terminal drop in pressure was obscure. There was no clear indication that it resulted from impairment of cardiac action. No dilatation of the heart or pulmonary edema was found at autopsy. In fact examination failed to reveal any significant lesions beyond those observed in animals sacrificed prior to the terminal phase. It is unlikely, and at least not proven, that the cause was abrupt capillary stasis, either generalized or local. Through reduced blood flow and progressive anoxia in tourniquet shock, there is profound tissue injury with impairment of vital functions but exactly how this relates to sudden decline in arterial pressure and death is not clear.

Discussion. We are unaware of previous studies of arterial pressure curves in rats with tourniquet shock. Pressures generally resembling those in this study have been noted in dogs with tourniquet(3,4) or compression shock(5) and also in dogs with plasmapheresis(6).

The arterial pressures following release of a unilateral 5 hour tourniquet are of interest in connection with the problem of irreversibility. We have shown that this fulminant form of shock is not characterized by an irreversible stage. To the contrary it is reversible via fluid therapy, with or without replacement of the tourniquet, even up to the time of death (7,8). In addition, evidence is available that no bacterial factor is involved(9).

It might be said that this type of shock lacks irreversibility because it is not associated with drastic hypotension. Throughout most of tourniquet shock in our study the arterial pressure remained above 60 mm Hg. Blood pressure becomes especially pertinent when the tourniquet and hemorrhagic forms of shock are compared, since extremely low pressures, *i.e.* 30 to 45 mm Hg, maintained over a period of time, often form the basis of standard irreversible hemorrhagic shock(10, 11). Such technics, which involve loss of whole blood rather than plasma, presumably give more marked anoxia and tissue injury than occur in tourniquet shock and this might be a critical factor in the development of an irreversible state.

Regardless of the validity of this argument, there is no doubt that profound tissue injury can result from marked and rapid loss of plasma. In fact such injury in the tourniquet and related procedures is clearly out of proportion to the degree of hypotension. For example it was shown in dogs with compression shock that systemic blood flow was seriously impaired even when arterial pressures exceeded the 50 to 70 mm Hg range(5). In dogs with acute plasmapheresis cardiac output declined more rapidly than the blood pressure and the discrepancy was more marked with loss of plasma than with comparable hemorrhage(6). Our own view is that arterial pressure is not a reliable criterion of either the severity or outcome of tourniquet shock.

As indicated, the shock we produce by release of a unilateral 5 hour tourniquet is of acute type and, if untreated, causes death within a few to several hours. Nevertheless, proper therapy will save even moribund animals. Successful reversal of the shock state can be obtained in the last few minutes of life, when arterial pressure is declining abruptly and the levels are about 30 to 40 mm. Approximately 70% of rats recover with fluid therapy alone(8) and nearly 100% with fluid plus replacement of tourniquet(7). This indicates that in the rat tourniquet shock is basically a reversible form of shock.

Summary. Arterial pressure in rats with fatal tourniquet shock shows 3 main phases: (1) an abrupt fall immediately after tourniquet release, (2) sustained hypotension within a relatively narrow range during most of the shock state, and (3) a terminal more or less rapid decline associated with death. Only in the terminal period does profound hypotension intervene, with levels below 40 mm. Although arterial pressure is not a reliable index of the severity of tourniquet shock, the absence of drastic hypotension might help explain why this form of shock is not irreversible.

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Removal of Blood Ammonia by Hemodialysis.* (22302)

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In this communication we wish to report our experience with hemodialysis performed on dogs with raised blood ammonia values.

The use of portacaval shunts for portal hypertension and the use of cation exchange resins have presented problems in ammonia metabolism resulting in a considerable revival of interest in the metabolism of amino acids and ammonia in liver disease(1,2). Many patients in hepatic coma and acute liver failure have demonstrated an increase in the value for ammonia in the blood(3). Elevated blood ammonia values have been found and also induced in some patients with portacaval shunts (anastomosis of the portal vein to the vena cava)(1). It seemed of interest therefore to see whether the Kolff artificial kidney would reduce the values for blood ammonia in patients and in experimental animals.

Materials and methods. A number of dogs have been subjected to hemodialysis using a modified Kolff dialyzer. Mongrel dogs weighing from 10 to 20 kg were prepared by producing an end to side portacaval shunt. The "meat intoxication" syndrome was induced by feeding of meat and urea as described by Riddell(4). Eight dogs with an elevated blood ammonia level and "meat intoxication" manifested by lethargy, blindness, ataxia, catatonia, or convulsions were treated with the artificial kidney for 3 hours using a flow of 100 ml per minute and a dialyzing surface of about 12,000 cm^2. Blood ammonia, measured by the microdiffusion technic of Conway (5) utilizing a 10 minute diffusion time(6), was measured at the beginning of the cellophane loops used for dialysis and 2 minutes later at the end of these loops, the determinations were made intermittently during the period of dialysis for the purpose of evaluating the rapidity of removal of blood ammonia.

Results. Percent removal of blood ammonia under these circumstances has been plotted against arterial concentration. (Fig. 1). It can be seen that blood ammonia concentration has been generally lowered by 50% during one passage through the artificial kidney with a tendency toward higher percentage removal with higher blood concentrations. The total amount of blood ammonia removed was estimated by multiplying the amount removed per milliliter of blood by the volume of blood dialyzed for successive twenty minute periods throughout the dialysis. This total amount removed was found to vary in 4 dialyses from 15,000 μg to 130,000 μg. When approximately 25,000 μg of blood ammonia had been removed, ammonia became detectable in the 100 liters of bath fluid used. No ammonia was ever detected in the bath fluid at the start of dialysis. The dif-

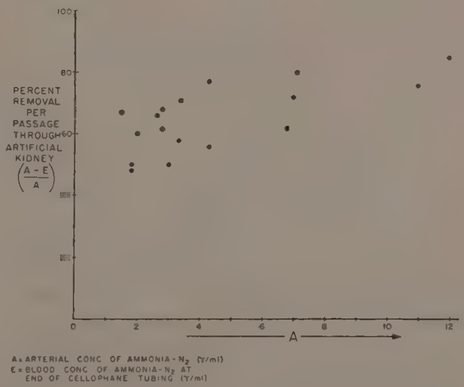

FIG. 1. Removal of ammonia from blood during one passage through a Kolff artificial kidney. Graph indicates % ammonia removed from blood of experimental animals with elevated values for blood ammonia. Note that the higher the value for blood ammonia the greater the extraction by dialysis. Normal value for blood ammonia in dogs is usually under 1 μg/ml. (Flow—100 ml/min.; dialyzing surface—approximately 12,000 cm^2.) (At a flow of 100 ml/min., clearance in ml/min. by the artificial kidney is numerically equal to percent removal.)

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* This work was supported by a grant from the U.S. Public Health Service.

fusion of ammonia out of the bath fluid into the air however, may make erroneous an estimation of ammonia removal based on the amount of ammonia in the bath fluid. No fall in blood ammonia was noted on passage of blood through the artificial kidney if the cellophane loops were not immersed in the bath fluid. In fact a small but definite rise in blood ammonia was noted under these circumstances. Normal dogs without portacaval shunts when subjected to hemodialysis in the same manner as those with meat intoxication did not show any appreciable elevation of ammonia in the blood entering or leaving the kidney.

Comment. The invariable lowering of blood ammonia concentrations during passage through the artificial kidney and the appearance of ammonia in the bath water as dialysis proceeded indicated to us that hemodialysis removed blood ammonia in dogs with elevated blood ammonia levels.

We are presently using this method of removing blood ammonia to study certain aspects of liver disease. Whether hemodialysis may have any clinical value in the treatment of hepatic coma is also being investigated.

Summary. Blood ammonia, as measured by the microdiffusion technic of Conway, is removed efficiently from the blood of dogs with elevated blood ammonia levels by a Kolff type artificial kidney with a dialyzing surface of 12,000 cm^2. When flow through artificial kidney is 100 ml/minute the clearance of blood ammonia varies from about 50 ml/minute at lower arterial concentrations of blood ammonia to about 80 ml/minute at higher arterial concentrations.

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Hypothalamic Stimulation of ACTH Secretion. (22303)

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It has been shown that antidiuretic and oxytocic hormones of the hypothalamic-posthypophyseal system* may stimulate the anterior lobe of the pituitary gland to discharge ACTH. It has been demonstrated that these hormones produce, when injected into normal animals, significant fall of circulating eosinophils(2), adrenal ascorbic acid (3) and cholesterol(4); these actions which have not been observed in hypophysectomized animals(3,4), are apparently due to stimulation of the adenohypophysis. Release of ACTH occurs also in hypophysectomized rats bearing a hypophyseal graft in the anterior chamber of the eye after local injection of small amount of posterior pituitary hormones into the subconjunctival space(5). This observation rules out the possible participation of a "stress" reaction in the ACTH-releasing power of posterior pituitary hormones. These results are in agreement with the hypothesis(2-6) that hormones produced in the hypothalamic nuclei may act as neurohumoral agents in the hypothalamic control of the activity of the anterior lobe of the pituitary gland.

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* There is much positive evidence to support the view that "posterior pituitary hormones" are produced by cells of the nuclei supraopticus and paraventricularis and pass along the axons of the tractus supraoptico-hypophyseus into the posterior lobe which serves as a storage-release center(1).

TABLE I. Effect of Continuous Injection of Antidiuretic (Pitressin, Parke-Davis) and Oxytocic Hormones (Pitocin, Parke-Davis) on Mean Weight of Body, Hypophysis and Adrenal Glands in Normal Rats; 10 Rats in Each Series, Treated 15 Days.

Treatment	Body wt (g) Initial	Body wt (g) Final	Hypophysis wt (mg/100 g final body wt)	Adrenal wt (mg/100 g final body wt)
Pitressin (.5 U/rat)	148 ± 5	146 ± 8	4.41 ± .20	15.92 ± .8
Pitocin (.5 U/rat)	158 ± 7	154 ± 6	4.48 ± .21	15.02 ± .9
.9% NaCl	138 ± 5	145 ± 7	4.66 ± .30	11.76 ± .7
Inactivated Pitressin (.5 U/rat)	156 ± 8	162 ± 6	4.35 ± .20	10.85 ±1.1

The experiments here described confirm that both antidiuretic and oxytocic hormones may induce a conspicuous discharge of ACTH; these hormones produce, when chronically injected in normal rats, considerable modifications of adrenal weight and histology, which are prevented by hypophysectomy; injections of hormones previously inactivated with sodium thioglycollate are without any activity.

Methods. Normal and hypophysectomized male Sprague-Dawley rats, average weight 150 g, were used. All animals were given a commercial diet and drinking water *ad libitum.* Ten normal rats were given 0.5 U/rat of antidiuretic hormone (Pitressin, Parke-Davis) in daily subcutaneous injections, through 15 days; 10 rats were given 0.5 U/rat of oxytocic hormone (Pitocin, Parke-Davis) in same manner and time: 10 rats were daily treated with 0.5 U/rat of Pitressin previously inactivated in accordance with Van Dyke's thioglycollate method(7); 10 control rats were daily injected with 1 ml of 0.9% NaCl solution. An analogous experiment was carried out in rats hypophysectomized by the usual parapharyngeal approach. The rats were killed 24 hours after last injection. Pituitaries and adrenals were carefully dissected out and weighed. The pituitaries were then stained by Rasmussen-Ignesti's technic: the adrenals were stained with hematoxylin and eosin, with Sudan III and with Smith-Dietrich's method. The lipase content of adrenals was studied by Gomori's method.

Results. Body and organ weights of normal rats treated with posterior pituitary preparations are shown in Table I.

Neither antidiuretic nor oxytocic hormones cause significant changes in weights of body and anterior pituitary. After treatment with either antidiuretic or oxytocic hormones a conspicuous and significant enlargement of the adrenal glands was observed: the inactivated hormone was completely ineffective.

The histological picture of the anterior pituitary gland after treatment with active Pitressin or Pitocin showed a higher incidence of basophilic cells. Adrenal glands of animals treated with active hormones showed a characteristic enlargement of the "zona fasciculata" and a conspicuous increase of sudanophilic material (Fig. 1 and 2); lipasic activity was also enhanced. These effects were not obtained with the inactivated preparation.

In hypophysectomized rats the posthypophyseal extracts did not produce any modification of weight and of the histology of the suprarenal glands (Table II).

Discussion. The results of these experiments completely agree with Moehlig's(8) and Hantschmann's(9) observations; these authors have described adrenal hypertrophy in rabbits after treatment with whole posterior pituitary extract; Heinbecker's(10) observation that posterior pituitary hormones

TABLE II. Effect of Continuous Injection of Antidiuretic (Pitressin, Parke-Davis) and Oxytocic Hormones (Pitocin, Parke-Davis) on Mean Weight of Body and Adrenal Glands in Hypophysectomized Rats; 10 Rats in Each Series, Treated 15 Days.

Treatment	Body wt (g) Initial	Body wt (g) Final	Adrenal wt (mg/100 g final body wt)
Pitressin (.5 U/rat)	165 ± 7	155 ± 8	9.11 ± .9
Pitocin (.5 U/rat)	170 ± 9	158 ± 7	8.20 ± .7
.9% NaCl	168 ± 7	157 ± 8	9.02 ± .9

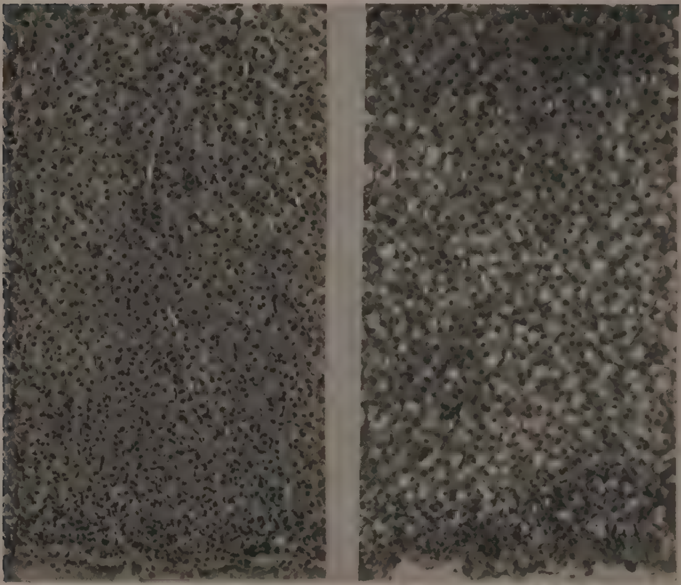
Fig. 1 Fig. 2

Adrenal cortices fixed in 10% formalin and stained with hematoxylin and eosin.

FIG. 1. Adrenal cortex of control rat.
FIG. 2. " " " rat treated with antidiuretic hormone through 15 days.

may induce a marked basophilism in the pituitary gland is also confirmed.

The evidence herein presented supports earlier observations(2-6) that both oxytocic and antidiuretic hormones can activate the adrenal cortex by causing the release of adrenocorticotrophic hormone; experiments in hypophysectomized rats eliminate the possibility that the effects observed may be due to contamination with ACTH of the injected hormones. Complete inefficacy of the inactivated posterior pituitary preparation seems to indicate that the ACTH-releasing power is strictly linked to the posterior pituitary hormonal molecule and not to an impurity contained in posterior pituitary extracts as suggested by Saffran(11).

These experiments strongly suggest that the "posterior pituitary hormones" or some related compounds may be considered as a neurohumoral agent linking the hypothalamus to the anterior pituitary through the portal blood flow(12). Considerable additional evidence is now available in favour of this hypothesis. Green(13), Palay(14) and Scharrer(1) have found considerable Gomori-positive neurosecretory material, (containing posterior lobe hormones) in the proximity of the hypophyseal portal vessels, in the median eminence and in the infundibular stem. Rothballer(15) and Barrnett(16) have shown that after exposure of rats to pain stimuli the neurosecretory material of the hypothalamo-hypophyseal system is depleted from the median eminence and from the infundibular process into the portal vessels. Mirsky(17) has reported an acute elevation in blood antidiuretic hormone levels after various stimuli, such as pain and adrenalectomy, all associated with augmented ACTH discharge. McCann(18) observed in rats that hypothalamic lesions, which produce diabetes insipidus, uniformly block ACTH secretion; ACTH secretion appears to be produced in operated rats by Pitressin administration. Sobel(19) demonstrated that Pitressin causes an increased excretion of urinary corticoids by guinea pigs.

Summary. 1) Both antidiuretic and oxytocic hormones of the hypothalamic-posthypophyseal system produce, when chronically injected in normal rats, a significant enlargement of the adrenal glands and a conspicuous increase of the adrenal sudanophilic material. 2) The hormones do not produce modification in hypophysectomized animals. 3) An inactivated preparation of antidiuretic hormone is completely ineffective. 4) These results are consistent with the hypothesis that antidiuretic and oxytocic hormones of the hypothalamo-posthypophyseal system may act as neurohumoral agents linking the hypothalamus to the anterior pituitary through the portal blood flow.

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Actions of Reserpine and Chlorpromazine Hydrochloride on Rat Brain Oxidative Phosphorylation and Adenosinetriphosphatase.* (22304)

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The use of chlorpromazine and reserpine in treating psychiatric disorders has been discussed by several investigators(1-8). Chlorpromazine is widely used as a tranquilizing agent for mentally disturbed patients and is regarded as a general central nervous system depressant capable of potentiating certain other depressant drugs(9-11). The sedative and hypotensive actions of reserpine have been attributed to a central inhibition of the sympathetic nervous system(12-14). Although chlorpromazine and reserpine are similar in their clinical actions, a number of opposing pharmacological characteristics have been reported. In contrast to chlorpromazine, reserpine facilitates production of convulsions by caffeine or metrazol and antagonizes actions of barbiturates and anti-convulsive drugs(15-17). Chlorpromazine-HCl, at concentrations of 10 mg% (2.8 x 10^{-4} M) or greater, has been reported to depress oxygen uptake by brain slices(18).

In the present investigation, effects of chlorpromazine and reserpine upon two important high energy phosphate enzymatic reactions were studied. These compounds were tested separately and in combinations in order to detect possible synergistic or antagonistic actions. For comparison chlorpromazine was tested in combinations with sodium pentobarbital, the latter drug also being known to uncouple oxidative phosphorylation(19).

Materials and methods. Adult Sprague-Dawley albino rats were used. Fifteen percent brain homogenates were prepared in isotonic sucrose with a Potter-Elvehjem type

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TABLE I. Effect of Reserpine on Oxidative Phosphorylation in Rat Brain Homogenates.

Reserpine, μg/ml	Δ P		Δ O		P : O	
	μM	% ± S.E.	μatoms	% ± S.E.	Ratio	% ± S.E.
0	27.2	100	10.11	100	2.70	100
10	27.0	99.2 ± 1.6	10.07	99.6 ± 1.1	2.68	99.5 ± .8
25	25.5	93.4 ± 1.2†	9.70	95.9 ± 1.6*	2.63	97.6 ± 1.7
50	23.9	87.8 ± 1.8†	9.05	89.6 ± 1.8†	2.64	98.2 ± 3.1

Δ P = μmoles inorganic phosphate esterified. Δ O = μatoms oxygen uptake.
* p $<$.05 and † $<$.01 that differences from controls are due to chance.

homogenizer. The components added to each Warburg flask for the study of oxidative phosphorylation were as follows: in the main compartment, 100 μM of KCl, 25 μM of glycylglycine, 40 μM of K_2HPO_4, 3 μM of KOH, 0.03 μM of cytochrome c, 26 μM of Na-pyruvate, 4 μM of malic acid, 2.5 μM of K-ATP (neutralized with KOH), 16 μM of $MgCl_2$, 20 μM of NaF, and 75 mg of rat brain homogenate in a total of 1.8 ml of triple distilled water; in the sidearm, 5 mg of hexokinase (Pabst) and 50 μM of dextrose in a total of 0.2 ml; and in the center well, 0.2 ml of 10% KOH. Additions of drugs† are indicated in appropriate tables. Because of its insolubility in water, reserpine was dissolved in 10% polyethylene glycol containing 0.04 malic acid and 0.26 M Na-pyruvate, and all concentrations of the drug were added to the flasks in a total of 0.1 ml containing the substrates, so that in experiments with reserpine all flasks including controls contained 0.01 ml of polyethylene glycol as well as the components mentioned above. The vessel contents when mixed was pH 7.4. All steps directly involving the homogenate were carried out in an ice water bath until the vessels were attached to the manometers and then placed in the incubating bath. The sidearms were tipped after 6 minutes, and zero time readings were taken exactly 3 minutes after tipping each flask. Zero time flasks were removed and 0.2 ml aliquots were quickly pipetted into 2.8 ml of 0.32 M trichloracetic acid. Oxygen uptake of the experimental flasks was measured for 25 minutes at 25°C, after which 0.2 ml aliquots were also added to trichloracetic acid. Inorganic phosphate uptake was determined for each set of experimental conditions(20) and P:O ratios were calculated. The method of DuBois and Potter(21) was employed for ATPase determinations. In experiments with reserpine, the drug was dissolved in 10% polyethylene glycol containing 0.02 M malic acid, and all test amounts were added in 0.05 ml. All flasks including controls,

TABLE II. Effects of Combinations of Reserpine and Chlorpromazine on Oxidative Phosphorylation in Rat Brain Homogenates.

Chlorpromazine, M × 10^4	Reserpine, μg/ml	Δ P			Δ O			P : O		
		μM	% inhibition by RSP ± S.E.	p*	μatoms	% inhibition by RSP ± S.E.	p*	Ratio	% inhibition by RSP ± S.E.	p*
0	0	26.3			9.50			2.77		
	50	24.2	7.9 ± 1.3	—	8.42	11.2 ± 2.0	—	2.88	−4.0 ± 1.4	—
2.5	0	23.5			8.56			2.75		
	50	18.7	20.4 ± 1.4	$<$.001	7.22	15.6 ± 2.7	$>$.1	2.60	5.2 ± 2.4	$<$.01
5.0	0	16.3			6.78			2.41		
	50	9.7	41.0 ± 2.0	"	4.61	31.5 ± 3.9	$<$.001	2.11	12.5 ± 3.5	$<$.01
7.5	0	6.8			4.14			1.64		
	50	2.9	57 ± 4	"	2.59	37.1 ± 3.4	"	1.10	32.3 ± 5.3	$<$.001

RSP = Reserpine.
* p values indicate significance of inhibition by reserpine in the presence of various concentrations of chlorpromazine as compared with reserpine inhibition in the absence of chlorpromazine.

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† Chlorpromazine hydrochloride and reserpine were kindly supplied by Smith, Kline and French Laboratories, Philadelphia, Pa., and Ciba Pharmaceutical Co., Summit, N. J., respectively.

TABLE III. Effects of Combinations of Sodium Pentobarbital and Chlorpromazine on Oxidative Phosphorylation in Rat Brain Homogenates.

		Δ P			Δ O			P:O		
Chlorpromazine, M × 10^4	Pentobarbital, M × 10^4	μM	% inhibition by PTB ± S.E.	p*	μatoms	% inhibition by PTB ± S.E.	p*	Ratio	% inhibition by PTB ± S.E.	p*
0	0	28.4			10.59			2.69		
	2.5	15.1	46.9 ± .9	—	5.92	44.0 ± .9	—	2.55	5.0 ± 1.5	—
2.5	0	23.6			9.32			2.53		
	2.5	12.2	48.3 ± 1.4	>.1	5.00	46.4 ± 1.0	>.1	2.44	3.5 ± 3.1	>.1
5.0	0	14.9			7.22			2.06		
	2.5	7.4	50.5 ± 1.6	>.05	3.76	47.9 ± 2.1	"	1.98	3.6 ± 5.3	"
7.5	0	6.5			4.56			1.43		
	2.5	2.9	55.3 ± 3.3	"	2.67	41.0 ± 2.9	"	1.10	23.1 ± 6.7	<.05

PTB = Pentobarbital.

* p values indicate significance of inhibition by pentobarbital in the presence of various concentrations of chlorpromazine as compared with pentobarbital inhibition in the absence of chlorpromazine.

therefore, contained 0.005 ml of polyethylene glycol and 1 μM of malic acid as well as the other necessary components. Sufficient KOH was added to the stock solution of ATP to bring the reaction mixture to pH 7.45.

Results. All of the studies represent determinations from 6 or more animals each. At flask concentrations of 25 and 50 μg/ml of reserpine, small but significant decreases in phosphate and oxygen uptake were observed (Table I), but no significant changes were found in P:O ratios(22).

The depression of oxidative phosphorylation of brain homogenate systems by chlorpromazine is shown in Tables II and III. Effective concentrations are similar to those reported by Finkelstein, Spencer, and Ridgeway(18), but are 10 times as high as those reported by Abood who used mitochondrial preparations(23). Reserpine at 8.22 x 10^{-5} M (50 μg/ml) more effectively depressed phosphate and oxygen uptake and P:O ratios in the presence of increasing amounts of chlorpromazine than in the absence of the latter drug (Table II). On the other hand, very little difference was observed in the percent depression of P, O, and P:O values by 2.5 x 10^{-4} M Na-pentobarbital in the absence and presence of increasing amounts of chlorpromazine (Table III).

Six rats were given 10 mg/kg of chlorpromazine-HCl subcutaneously and 6 control animals were given water injections. All were sacrificed after 1 hour. Animals receiving the drug were severely depressed and incontinent but were not unconscious. Oxidative phosphorylation by brain homogenates was determined in the absence and presence of Na-pentobarbital in the flasks. Small but significant increases in oxygen uptake were obtained with homogenates from animals treated with chlorpromazine in the absence and presence of 2.5 x 10^{-4} M pentobarbital in the flasks (Table IV). No changes were seen in organic phosphate uptake or P:O ratios.

Chlorpromazine up to 6.25 x 10^{-4} M resulted in little or no depression of hexokinase activity(24) so that the decrease in phosphate uptake was not due to depression of the hexokinase trapping system.

TABLE IV. *In Vivo* Chlorpromazine on Oxidative Phosphorylation in Rat Brain Homogenates.

Inj. chlorpromazine, mg/kg	Pentobarbital in flasks, M × 10^4	Δ P μM ± S.E.	Δ O μatoms ± S.E.	P:O Ratio ± S.E.
0	0	28.3 ± .4	10.19 ± .14	2.78 ± .03
	2.5	13.1 ± .7	5.37 ± .09	2.44 ± .11
10	0	28.5 ± .4	10.74 ± .07*	2.69 ± .04
	2.5	14.1 ± .4	5.78 ± .07*	2.44 ± .08

* p <.01 that increases over corresponding values from untreated animals are due to chance.

Reserpine up to 50 μg/ml (8.22×10^{-5} M) had no significant effect on rat brain adenosinetriphosphatase activity, and chlorpromazine at 1.2×10^{-4} M and 1.5×10^{-4} M only slightly depressed ATPase.

Discussion. Both chlorpromazine and reserpine are capable of depressing oxygen and inorganic phosphate uptake by rat brain homogenates, but at concentrations much higher than ordinary pharmacological or therapeutic doses. It is highly improbable that reserpine depresses phosphorylation *in vivo*, since single effective pharmacological doses of 1 mg/kg in animals and daily sedative doses of as little as 0.5 mg represent much lower concentrations than the 25 mg/l needed to depress phosphate and oxygen uptake in rat brain. It is interesting to note that in effective concentrations, reserpine acts synergistically with chlorpromazine in depressing phosphate and oxygen uptake and P:O ratios in brain homogenates in contrast to similar combinations of pentobarbital and chlorpromazine. Although the concentration of chlorpromazine (2.5×10^{-4} M or 89 mg/l) needed to depress oxidative phosphorylation in rat brain was much greater than the physiologically depressing dose of 10 mg/kg, small increases in oxygen uptake by brain homogenates from treated animals are suggestive that the drug may have some effect on oxidative processes *in vivo*. Small increases in rat brain oxygen uptake were reported to be insignificant by Grenell, Mendelson, and McElroy (26). However, increases in oxygen uptake have been reported by Abood(23) with mitochondria using 2×10^{-5} M (7.1 mg/l) chlorpromazine *in vitro*. This concentration is comparable to the usual pharmacological doses.

From the data presented here and in recent reports(18,19,23), it appears that mitochondrial preparations are more sensitive to certain depressant drugs than whole homogenates or slices. Studies by Johnson and Ackerman (25) showing that nuclei potentiate mitochondrial oxidative phosphorylation suggest the possibility that nuclei and perhaps other cell fractions may protect mitochondria from the actions of uncoupling drugs. Suitable studies of this subject are now being undertaken.

Summary. Oxidative phosphorylation by rat brain homogenates was depressed by chlorpromazine at 2.5×10^{-4} M or greater, while reserpine at 25 and 50 μg/ml only slightly depressed oxygen uptake and phosphorylation. Effective concentrations of reserpine appeared to depress phosphorylation synergistically with chlorpromazine. Oxygen uptake was slightly increased with brain homogenates from animals treated with 10 mg/kg or chlorpromazine. Reserpine up to 50 mg/l had no effect on enzymatic ATP breakdown, while chlorpromazine, at 1.2×10^{-4} M and 1.5×10^{-4} M depressed rat brain adenosinetriphosphatase slightly.

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Transmethylation Reactions *in vivo* and *in vitro* in the Young Calf.[*] (22305)

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Johnson *et al.*(1) have shown that the young calf on a "normal" methionine intake requires a dietary source of choline and the present investigation was undertaken to determine whether this was replaceable by methionine. Earlier work has shown that rat liver slices and homogenates methylate homocysteine to form methionine with either choline or betaine as methyl donor. In studies by Borsook and Dubnoff(2), guanidoacetic acid was shown to be methylated by methionine in guinea pig liver homogenates. Bernheim and Bernheim(3) demonstrated that rat liver homogenates could oxidize acetylcholine, but found guinea pig liver to be inactive in this oxidation. Kensler and Langemann(4) reported that human and rabbit liver resembled guinea pig liver in this respect. We have now studied these enzyme systems in the calf.

Methods. Methionine-choline interrelationship. Male calves of various dairy breeds, 24 hours old, were fed a synthetic milk diet previously described by Hopper and Johnson (5) except that alpha protein was used as the protein source in the diet. Various levels of DL-methionine were added to the diet at time of feeding to give the total dietary methionine values indicated in Table I. The vitamin premix contained all the vitamins, except choline, which was a variable in the experiment. When choline was administered, it was a constituent of the vitamin premix. All animals were fed *ad lib.* twice daily from nipple pails. At the end of the trials, all animals were sacrificed and liver samples obtained for ether extract determinations. Post mortem examinations of all animals were conducted for the detection of histological lesions.

In vitro studies using calf liver homogenates. Liver samples were obtained from calves which had been fed the complete synthetic milk diet and compared with liver samples from 175-200 g, male rats fed commercial laboratory pellets. These samples were handled and the study of the methylation of homocysteine by betaine was conducted by

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[†] This paper is taken from a thesis submitted by the senior author to the graduate college, University of Illinois in partial fulfillment of requirements for the Degree of Doctor of Philosophy in Animal Nutrition. Present address: Department of Animal Husbandry, University of Nebraska, Lincoln.

TABLE I. Effect on Various Levels of Dietary Methionine on Growth and Liver Ether Extract of Animals on Choline-Deficient Diets, in 15 Calves.

Days on trial	Total dietary methionine, %*	Dietary choline, %	Daily gain, lb	% ether extract in liver (dry matter basis)
35	.4	0	.26	9.75
35	”	0	.19	3.65
43	”	0	.35	6.42
41	”	0	.34	4.17
38	.8	0	.66	38.96
35	”	0	.86	23.32
44	1.2	0	.91	13.65
43	”	0	.70	13.41
33	”	0	1.00	9.52
33	”	0	1.40	18.00
41	1.6	0	.34	37.68
41	”	0	.27	26.57
41	.8	.2	.90	14.04
41	”	”	.54	4.90
33	”	”	1.20	9.56

* The basal ration contained 0.3% methionine, and DL-methionine was added to give these total values.

procedures outlined by Williams *et al.*(6) and by Mistry *et al.*(7). The methylation of guanidoacetic acid by methionine was studied according to the method of Borsook and Dubnoff as used by Mistry *et al.*(7). Choline oxidase activity was determined by the method of Richert and Westerfeld(8).

Results. In vivo studies. As noted in Table I, good growth was observed in the calves receiving the choline-deficient diets containing 0.8 or 1.2% DL-methionine. However, the livers of the calves receiving only 0.8% DL-methionine contained a considerably greater amount of ether extract. This indicates a deficiency of labile methyl groups at this level of methionine in the absence of choline. Treadwell(9) has reported that the young rat has a definite need for choline when the diet contains 0.8% methionine. Neumann *et al.*(10) found the young pig required 0.1% choline when fed a "synthetic milk" ration containing 0.8% methionine. The calves receiving a total of 0.4% methionine in the diet‡ and no choline exhibited very small daily gains. This result was due to a methionine deficiency as well as a deficiency of labile methyl groups and the extremely poor growth presumably accounts for the failure to find choline-deficiency fatty livers. Diets supplying a total of 1.6% methionine supported very poor growth and liver samples from these animals during the feeding trial indicated that the 1.6% level of methionine was apparently "toxic," as the animals scoured severely throughout the trial. Liver ether extract data for the animals receiving 1.2% methionine, are similar to those from the normal animals receiving 0.8% methionine and 0.2% choline, as seen in Table I. Histological study of liver revealed fatty infiltration and some kidney damage in the methyl-deficient animals, confirming the liver fat data. The results indicate that the young calf can, by transmethylation, synthesize its required choline from methionine as has been shown for the rat, for the pig, and for man.

In vitro studies. The results of methionine formation by calf liver homogenates are shown in Table II. Although there was some transmethylase activity in calf liver homogenates, the activity was much less than that of rat liver homogenates even though both species were on diets which were satisfactory for growth.

The results of creatine formation by calf liver homogenates are presented in Table II. Under the same experimental conditions, over 4 times as much creatine was formed by rat liver homogenates as by calf liver homogenates.

TABLE II. Results of Enzyme Studies in Calf and Rat Liver Homogenates.

	No. of animals	Results
Methionine synthesis from homocysteine + betaine		Avg methionine formed/100 mg dry matter of liver tissue/hr, μg
Calf	2	259
Rat	3	[illegible]
Creatine synthesis from guanidoacetic acid + methionine		Avg creatine formed/100 mg dry matter of liver tissue/hr, μg
Calf	2	6.9
Rat	2	29.6
Choline oxidase activity		μl/O_2/10 min./flask
Calf	2	[illegible]
Rat	2	[illegible]

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‡ This diet also contained approximately 0.15% cystine, supplied in the alpha protein.

nates. However, both species were able to methylate guanidoacetic acid to form creatine. The low values found in this study for calf liver are of the same magnitude as those reported by Cohen(11) for beef liver. Cohen (12) found that addition of folic acid and glutamic acid did not bring the levels into line with those for the rat.

The choline oxidase activity of calf liver homogenates as compared to rat liver homogenates is shown in Table II. These results indicate that calf liver possesses a much lower choline oxidase activity than rat liver. The low activity of the enzyme would indicate a slow choline turnover similar to that found by Handler(13) for the guinea pig.

Summary. 1. Using a synthetic milk diet, calves receiving 1.2% DL-methionine exhibited no demonstrable choline requirement, as measured by growth and liver ether extract. On a diet supplying 0.8% methionine, fatty livers developed in the absence of choline. 2. A study was made of the ability of calf liver homogenates to methylate homocysteine to form methionine, when betaine served as methyl donor; to methylate guanidoacetic acid, when methionine served as the methyl donor, and to oxidize choline. It was found that while calf liver homogenates would carry out all of these reactions, the activity of the calf liver enzymes was very much less than that of rat liver homogenates.

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Pernicious Anemia. I. Remission by Small Oral Doses of Purified Vitamin B_{12}.* (22306)

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During studies on pernicious anemia, it was found, contrary to previous experience, that partial remission occurred in a patient with pernicious anemia in relapse who received minimal doses of purified vit. B_{12} by mouth. Pernicious anemia is generally considered to be the result of a heredofamilial atrophy of gastric and duodenal mucosa, with absence of "intrinsic factor," a material necessary for absorption of vit. B_{12} from the gastrointestinal tract into the blood stream. Megaloblastic erythropoiesis of pernicious anemia in relapse can be restored to normal by overcoming the deficiency in intrinsic factor by (1) parenteral administration of vit. B_{12}, thus circumventing the abnormal gastrointestinal tract; (2) oral administration of massive doses of vit. B_{12} (1000 to 9000 μg/wk), through a sort of "mass action" effect; or (3) oral administration of vit. B_{12} together with some outside source of intrinsic factor. How-

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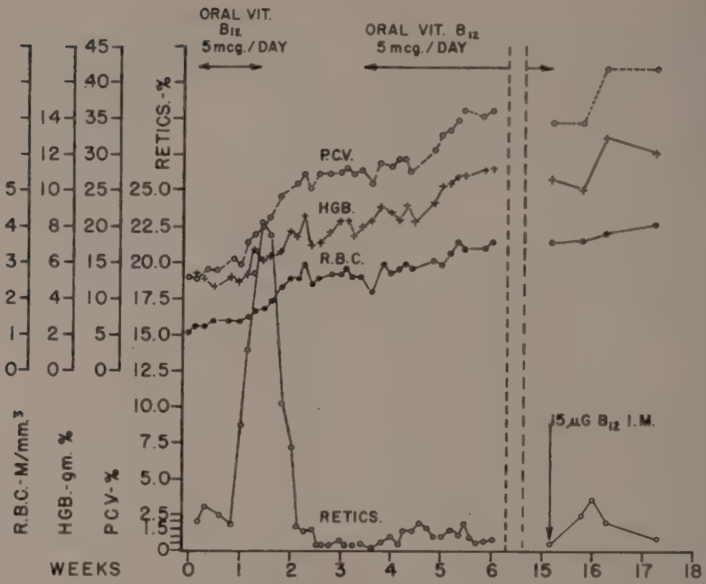

FIG. 1. Case 1a. Pernicious anemia. Submaximal response following oral administration of vit. B_{12}, 5 μg daily.

ever, remissions of pernicious anemia following oral administration of small amounts of vit. B_{12} without intrinsic factor (10 to 250 μg/day) have been reported(1-7). Suboptimal responses have been seen with oral doses of 10 μg(1,2), 15 μg(3), 30 μg(2), and 50 to 250 μg per day(1,4-6); but only rarely have maximal responses to oral vit. B_{12} in doses of 25 to 150 μg/day been noted(1,4,6). In the absence of an outside source of intrinsic factor, remission following oral vit. B_{12} occurred regularly only when massive doses of the material (3000 μg in a single dose) were given (8).

As a result of our experience with one patient (Fig. 1), in whom pronounced reticulocytosis and remission followed oral administration of 5 μg/day of purified vit. B_{12} without added intrinsic factor, similar small doses of vit. B_{12} (5.0 to 16.8 μg/day) were given by the same route to 9 additional patients with pernicious anemia in relapse, and to 1 patient with megaloblastic anemia of obscure etiology. It is our purpose to report the almost regular occurrence of varying degrees of remission under these circumstances.

Methods. Diagnosis of pernicious anemia was established by the presence of macrocytic anemia, histamine-fast achlorhydria, megaloblastic bone marrow, normal gastrointestinal x-ray studies, and absence of steatorrhea. The dose of 5.0 to 16.8 μg of vit. B_{12} was administered by mouth, one to 3 times daily, in tablets or capsules, with no particular relation to meals(6). The vit. B_{12} was obtained from 2 independent sources,†, and was assayed for and found to be free of folic and folinic acids. No intrinsic factor was given in association with oral vit. B_{12}, and the diet, which was free of liver and red meats, was unchanged before and during treatment.

Results. A definite reticulocytosis and hematologic remission of varying degree, with reversion of bone marrow to normoblastic erythropoiesis, occurred in 11 trials in 8 cases of pernicious anemia and in one case of nonpernicious megaloblastic anemia (Table I).

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† We are indebted to Dr. Douglas B. Remsen, Squibb Institute for Medical Research, New Brunswick, N. J., and to Dr. Nathaniel S. Ritter, Merck & Co., Rahway, N. J., for generous supplies of these materials.

TABLE I. Remissions in Pernicious Anemia (Cases 1-8) and Non-Pernicious Megaloblastic Anemia (Case 9) on Small Oral Doses of Vit. B_{12}.

Case	B_{12}/day, µg	Initial count Hgb, g/100 ml	 Hct, %	Max retics, %/day	Max hemogram Hgb, g/100 ml	 Hct, %
1 a	5	5.2	13	22.6/ 8	11.4	37
b	10	9.0	27	6.2/ 9	12.6	42
2 a	16.8	5.6	18	9.8/ 3	10.9	37
b	15	8.0	26	9.2/ 8	10.7	36
3	16.8	9.1	27	9.2/ 9	13.3	41
4	15	8.2	26	8.8/20	14	42
5 a	15	8.5	28	9.8/ 9	14.4	43
b	15	9.0	28	8.8/13	14.9	47
6	15	7.6	22	18.0/ 8	11.7	37
7	15	5.4	18	21.0/ 9	10.2	34
8	10	9.3	30	7.4/12	14.1	46
9	10	5.4	17	21.6/15	12.9	46
	Mean*	7.7	23.9	10.7/10	12.6	40.2
	Range*	5.2–9.3	13–28	6.2–22.6/3–20	10.2–14.9	34–47

* Of cases 1-8 only.

Remission was estimated to have been maximal in 3 cases of pernicious anemia and in the one case of non-pernicious megaloblastic anemia, and submaximal in 5 cases of pernicious anemia. Typical responses to oral doses of vit. B_{12} are depicted in Fig. 1-3, and the 9 responsive cases are summarized in Table I. Maximal reticulocytosis usually occurred on 8th or 9th day after initiation of therapy, with a cumulative oral dose of from 35 to 150 µg of vit. B_{12}. Unless parenteral B_{12} was subsequently administered, remission after cessation of therapy usually tended to be short-lived (4 to 6 months), although remissions persisted longer in some patients.

During these observations, 3 patients with pernicious anemia similarly treated failed to show hematologic response (Table II). Case 4 initially showed no hemoglobin response, despite a reticulocytosis, on a dose of 5.6 µg/day, but responded maximally on a dose of 15 µg/day (Table I). Six months later, when again in relapse, this patient failed to show any signs of remission on oral vit B_{12} during

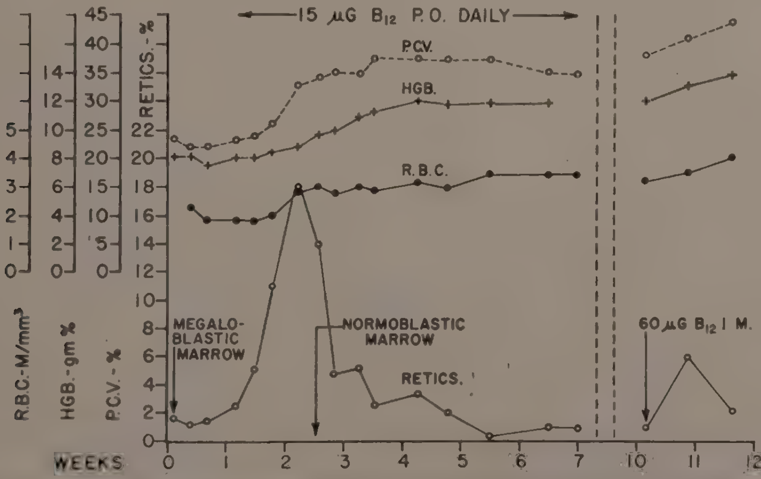

FIG. 2. Case 6. Pernicious anemia. Submaximal response following oral administration of vit. B_{12}, 15 µg daily.

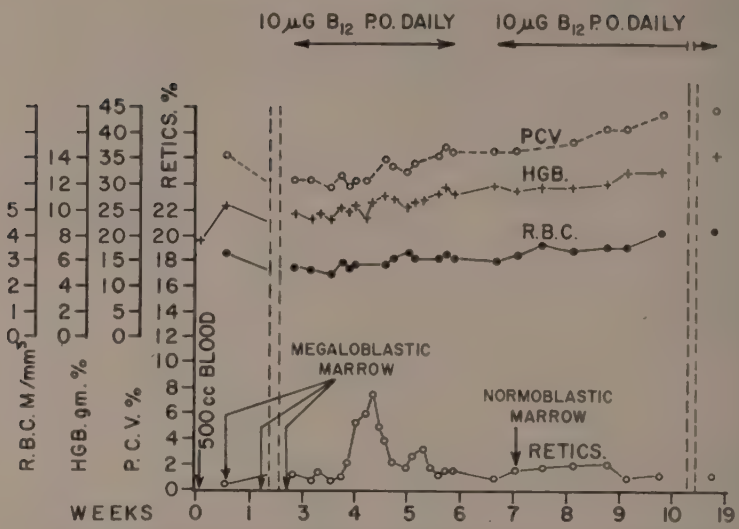

FIG. 3. Case 8. Pernicious anemia. Maximal response following oral administration of vit. B_{12}, 10 µg daily.

successive trials on 5, 10 and 15 µg/day, but at this time he had developed carcinoma of the stomach. Case 7, who showed a remission on the initial oral medication of 15 µg daily (Table I), failed to respond to a similar oral dose following another relapse 9 months later (Table II), whereas parenteral vit. B_{12} was effective. No response to 5 µg of vit. B_{12} was noted in one case of pernicious anemia in relapse (Case 10, Table II), but a larger dose and longer period of observation were not attempted in this patient.

Discussion. If intrinsic factor is indeed necessary for absorption of vit. B_{12}, it appears that some intrinsic factor must have been present in gastric and duodenal mucosa of our patients with pernicious anemia in relapse. Similar conclusions regarding the quantitative rather than qualitative change in gastric secretion in some cases of pernicious anemia were reached by others(9,10), who suggested that the red cell level in pernicious anemia is determined by actual amount of intrinsic factor secreted in the gastric juice.

Since crystalline vit. B_{12} was used in these studies, it is recognized that absorption may have been facilitated by the purified nature of the material, just as ferrous sulfate is absorbed more easily than food iron. This, however, is not a satisfactory explanation in itself, since regular remission with oral use of purified vit. B_{12} has not been reported(1-7).

TABLE II. Cases of Pernicious Anemia Which Failed to Respond to Oral Vit. B_{12}.

		Initial count			Max hemogram	
Case	B_{12}/day, µg	Hgb, g/100 ml	Hct, %	Max retics, %/day	Hgb, g/100 ml	Hct, %
4*	5.6	5.3	16.5	6.6/ 8	6.4	20
	5	8.4	27	4.8/18	8.4	27
	10	6.9	27	5.0/ 4	7.2	24
	15	7.0	23	5.4/26	7.0	23
7*	15	6.6	20.5	2.6/16	6.6	20.5
10	5	8.2	25	2.1/ 8	7.4	25
	Mean	7.1	23.2	4.4/13.3	7.2	23.3
	Range	5.3–8.4	16.5–27	2.1–6.6/4–26	6.4–8.4	20–27

* Same numbers as in Table I.

Although the fasting state may facilitate absorption of vit. B_{12}(6) from the gastrointestinal tract, absorption occurred independent of the fasting state in our patients.

The action of oral preparations containing both intrinsic factor and vit. B_{12} cannot *a priori* be attributed to their intrinsic factor content, since as has been shown above, remission may follow the use of the vit. B_{12} alone.

Studies on vit. B_{12} metabolism in this group of patients are still in progress. Preliminary results in some of these patients suggest that remission occurs without a rise of the serum vit. B_{12} level above relapse levels (less than 20 $\mu\mu$g/ml).

Summary. 1. Hematologic and clinical remissions were induced 11 times in 8 patients with pernicious anemia in relapse, and in 1 patient with non-pernicious megaloblastic anemia, by small oral doses of purified vit. B_{12} without added intrinsic factor. The effective dosage range was 5 to 16.8 μg per day. 2. Unequivocal failure occurred in 1 case of relapsed pernicious anemia. 3. These results further confirm the suggestion that in pernicious anemia there is a quantitative rather than a qualitative change in gastric secretion, and that variations in the amounts of intrinsic factor remaining in pernicious anemia patients may be responsible for varying responses to oral vit. B_{12}. 4. A trial period of vit. B_{12} alone should precede any study with a vit. B_{12} plus intrinsic factor preparation.

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Δ^1-Hydrocortisone: Plasma 17-Hydroxycorticosteroid Concentrations Following Oral and I. V. Administration.* (22307)

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During the past year the Δ^1-steroids have been employed clinically to a considerable extent. These compounds are synthetic derivatives of hydrocortisone and cortisone, differing from the parent steroids by the insertion of a double bond in the 1-2 position of the steroid nucleus. Δ^1-hydrocortisone ($\Delta^{1,4}$-pregnadiene-11β, 17α, 21-triol-3,20-dione) has been produced under various commercial names: metacortandralone, prednisolone, delta-cortef, hydeltra, meticortelone, and sterane.

Clinically, the Δ^1-steroids appear to be 3 to 5 times as effective as the parent compounds(1-3). A similar ratio has been found for glucocorticoid activity (liver glycogen deposition, eosinophil response)(3,4). However, results of *in vitro* studies of anti-inflammatory potency have not been reported. It is possible that the apparent advantages of the Δ-steroids over the parent compounds are attributable to differences in absorption or

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metabolism which permit more efficient utilization of the former steroids. This possibility has been investigated in the present study by measuring in human subjects the influence of oral or I. V. Δ¹-hydrocortisone administration on plasma 17-hydroxycorticosteroid (17-OHCS) concentrations.

Materials and methods. Normal young adults were the subjects used in all the following studies. In the study of oral administration each of 9 subjects was given a dose of 45 mg Δ¹-hydrocortisone§ orally. Plasma samples were obtained before and at 1, 2, 4, 6, and 8 hours after administration of the test dose and 17-OHCS concentrations determined by the method of Nelson and Samuels(5,6), using hydrocortisone as the reference standard. In another group of 6 subjects each was given a dose of 40 mg free hydrocortisone‖ orally and plasma samples were obtained for 17-OHCS determinations at 0, 2, 4 and 6 hours. In a third group of 11 subjects each was given 100 mg free hydrocortisone orally, and plasma samples were obtained at 0, 1, 2, 4 and 8 hours for 17-OHCS determinations. In the study of I. V. administration each of 8 subjects was given a dose of 0.5 mg/kg Δ¹-hydrocortisone intravenously as a 125 ml infusion over 30 minutes. Plasma samples for 17-OHCS concentration were obtained 1, 2, 3, and 4 hours after administration. From these data a regression line of best fit was determined by the method of least squares and the 17-OHCS half-life value calculated from the slope of the line.

Results. To determine whether the Δ¹-steroid was measured quantitatively by the Nelson and Samuels technic a comparison of the color development with the phenylhydrazine-sulfuric acid reagent was made using standard hydrocortisone and Δ¹-hydrocortisone. The resulting absorption curves for the two steroids were similar over a wave length range of 340-480 mμ, differing in that the curve for hydrocortisone had a greater density throughout (Fig. 1). After a correction factor for background influence was applied the density values at 410 mμ were essentially superimposed. The density values at 410 mμ for different amounts of steroid (Fig. 2) were shown to be the same for Δ¹-hydrocortisone as for hydrocortisone. Also, recovery following chromatography was almost the same, averaging 70% for Δ¹-hydrocortisone and 76% for hydrocortisone.

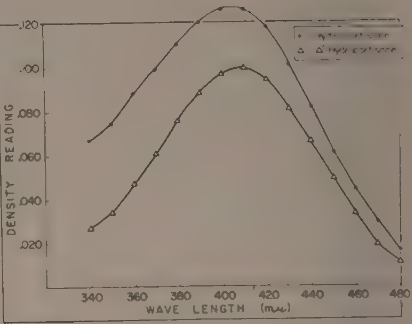

FIG. 1. Δ¹-hydrocortisone vs hydrocortisone: Absorption curves using phenylhydrazine-sulfuric acid reagent.

In Fig. 3 is shown a comparison of mean plasma 17-OHCS values at various time intervals following the oral administration of Δ¹-hydrocortisone and of free hydrocortisone. Following 45 mg Δ¹-hydrocortisone the peak 17-OHCS concentrations were greater than those following 40 mg free hydrocortisone, but less than those following 100 mg free hydrocortisone. In those subjects given 40

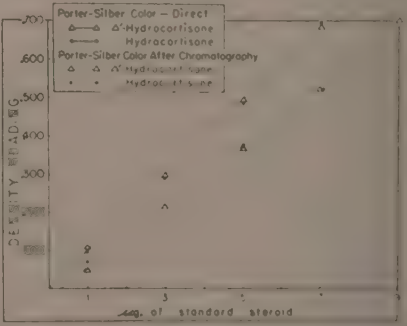

FIG. 2. Δ¹-hydrocortisone vs hydrocortisone: phenylhydrazine-sulfuric acid color development (410 mμ) directly and after column chromatography.

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§ C. T. Delta-Cortef® supplied through courtesy of Dr. C. J. O'Donovan, Upjohn Co., Kalamazoo, Mich.

‖ C. T. Cortef® supplied through courtesy of Dr. C. J. O'Donovan, Upjohn Co., Kalamazoo, Mich.

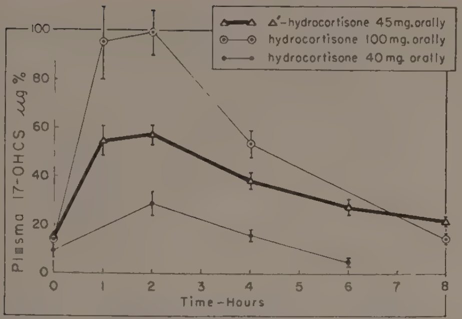

FIG. 3. Δ^1-hydrocortisone vs hydrocortisone: Plasma 17-OHCS concentrations following oral administration.

mg free hydrocortisone orally the plasma 17-OHCS concentrations were at or below control levels by 6 hours. A similar decline is seen in the curve representing the plasma 17-OHCS concentrations following the administration of 100 mg hydrocortisone orally, a return to normal values occurring by 8 hours. In contrast, the Δ^1-hydrocortisone curve falls more slowly; at 8 hours the mean 17-OHCS concentration is still well above the control value—and is significantly higher than the corresponding value following 100 mg hydrocortisone ($p < .01$).

The influence of intravenous administration of Δ^1-hydrocortisone on plasma 17-OHCS concentrations is indicated by the half-life data in Table I. The mean half-life obtained in 8 subjects was 192 minutes. In contrast to this, data reported from this laboratory(7) and elsewhere(8,9) for the mean half-life of hydrocortisone indicate that this is significantly less, ranging from 90 to 115 minutes. Thus, intravenous administration of Δ^1-hydrocortisone produces a more prolonged elevation of plasma 17-OHCS than does the intravenous administration of hydrocortisone.

Discussion. It has been shown that orally administered Δ^1-hydrocortisone, in doses roughly equivalent to those of free hydrocortisone, caused plasma 17-OHCS elevations both of greater magnitude and longer duration than did the latter steroid. When the dose of hydrocortisone was increased 2½ times the magnitude of 17-OHCS elevations became greater than that following Δ^1-hydrocortisone. However, even following the administration of this larger dose, these elevations disappeared more rapidly than those following the Δ^1-steroid. In addition, the 17-OHCS half-life in subjects receiving Δ^1-hydrocortisone intravenously was more prolonged than in those receiving hydrocortisone. These observations indicate that no appreciable differences in absorption exist but that the metabolism of Δ^1-hydrocortisone proceeds at a rate slower than that of hydrocortisone.

Results of *in vitro* experiments(10,11) are in accord with this interpretation. The biologically active adrenal corticosteroids contain the Δ^4-3-ketone structure in ring A, and in their metabolism this ring is reduced at carbons 4, 5, and then 3. The Δ^1-steroid must first undergo saturation of the 1-2 double bond to form the Δ^4 compound. Only following this is the Δ^4-3-ketone reduced to form the tetrahydro steroid and subsequently conjugated.

These data from both *in vivo* and *in vitro* experiments lend support to the possibility that differences in the rate of metabolism may account for the equivalent clinical effectiveness of smaller doses of the delta forms than of hydrocortisone or cortisone. To date, Δ^1-hydrocortisone represents the only steroid

TABLE I.

Plasma 17-OHCS Half-Life Values following Intravenous Δ^1-Hydrocortisone.

Normal patients	Half-life (min.)
D.C.	217
F.B.	189
T.H.	141
E.H.	205
J.K.	185
J.N.	232
L.O.	171
A.D.	197
Mean	192 ± 10

Plasma 17-OHCS Half-Life following Intravenous Hydrocortisone.

Author reference	No. normals	Mean half-life (min.)
Done(7)	9	90 ± 7.4
Brown(8)	11	111 ± 5.3*
Peterson(9)	18	115

* Calculated from author's individual 17-OHCS data.

which has been reported to have a half-life longer than that of hydrocortisone. Others studied include tetrahydro-hydrocortisone, 20-hydroxy-hydrocortisone, cortisone, and corticosterone(8,9); each of these has a half-life shorter than that of hydrocortisone. Of the steroids mentioned only cortisone, hydrocortisone, and Δ^1-hydrocortisone have a Δ^4-17, 21-dihydroxy-3,20-keto configuration and are potent glucocorticoids. Among these 3, the glucocorticoid potency and the half-life are related in such a way as to suggest that glucocorticoid potency is inversely proportional to the rate of metabolism.

Summary. The influence of oral or intravenous administration of Δ^1-hydrocortisone on plasma 17-OHCS concentrations was studied. With comparable oral doses Δ^1-hydrocortisone produced 17-OHCS elevations of greater magnitude and longer duration than did hydrocortisone. Also, the half-life of the Δ^1-steroid was longer than that of hydrocortisone. It was concluded that a slower metabolism of the Δ^1 form occurs and postulated that this might contribute to the greater clinical effectiveness of this steroid.

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A Non-hypophyseal Sex Difference in Estrous Behaviour of Mice Bearing Pituitary Grafts.* (22308)

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Differences in size of the pituitary gland related to sex have been described in some species, the female gland being larger and heavier than that of the male. Similarly, the gonadotrophic potency of injected hypophyseal extracts from males is greater than those prepared from female pituitaries(1,2). Castrated male guinea pigs with grafted ovaries showed a greater stimulation of the mammary glands and other sexual structures, than intact females(6). The same was true in castrated male mice bearing ovarian and vaginal grafts. There was a continuous non-cyclic stimulation of the vaginal tissue(5). However, Harris and Jacobson(3,4) have demonstrated that hypophysectomized female rats bearing male hypophyseal grafts from newborn donors, had normal estrous cycles, became pregnant and delivered their young at term. This would indicate that the characteristic cyclic gonadal function of the female, as compared to the non-cyclic of the male, would not be related to the sex of the pituitary, but rather to a sex difference in some other structure. Since functional pituitary grafts can be placed in hypophysectomized mice by means of a rather simple one-stage operation, we have used this animal preparation to investigate further the problem of estrous behaviour in hypophysectomized females and males bearing either male or female hypophyseal grafts, respectively.

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* Aided by grants from Minn. Division of Amer. Cancer Society and institutional grant from American Cancer Society.

Material and methods. Young mice of both sexes of the Z (C3H) stock and AZF_1 hybrids (A ♀ x Z ♂) were used. Hypophysectomy was performed according to the technic described by Thomas(7). The grafting of hypophyseal tissue was done as follows: The donor was killed by ether, the scalp and brain were lifted and the gland was removed and placed in a container with saline. Upon completion of hypophysectomy, the gland to be grafted was transferred to the emptied sella turcica of the host either with a fine pair of forceps or by holding the gland at the distal end of a very thin glass pipette by applying a slight negative pressure. The entire procedure was completed within 4 to 5 minutes and postoperative mortality did not exceed 10%. By this procedure, the overall incidence of successful hypophyseal grafts was 85% of a total of 325 operated animals for other studies. Mice bearing hypophyseal successful grafts have shown normal sexual activity throughout their lives (12 to 16 months). Two types of experiments were done. In one, the body weight changes were determined and the sexual cycles studied in 4 groups of female mice of the Z(C3H) strain, as follows: a) hypophysectomized; b) sham operated controls; c) hypophysectomized bearing a hypophyseal graft taken from a female donor of the same strain and age; d) hypophysectomized bearing a hypophyseal graft from a male donor of the same strain and age. In all groups the body weight was determined before and at weekly intervals after the operation. Similarly vaginal smears by the douche technic were taken daily during 1 week, at monthly intervals to determine the presence or absence of estrous cycles in the different groups. In another experiment, 2 groups of male AZF_1 hybrid mice were used. One of them was submitted to hypophysectomy followed by the graft of one hypophysis taken from a female donor. Another group carried their own (male) hypophysis. Three weeks after the operation, both groups of mice were castrated and 2 ovaries and a ring of vaginal tissue taken from female mice of the same strain and age were grafted subcutaneously, using the technic described by Huseby and Bittner(5). Finally, after a period of one month following the vaginal transplant, mice were killed, the grafts removed and studied histologically. The presence of hyperplasia and hypertrophy of the epithelial wall of the vaginal graft, and a complete cornification of the content of the lumen, without signs of cyclic activity, was interpreted as indicative of a continuous estrogenization(5).

Results. The results obtained in the first experiment, in which only animals showing successful takes of the grafts were included, are listed in Table I and can be summarized as follows: a) Sham hypophysectomized controls showed a progressive increase in body weight after the operation and, as was expected, normal estrous cycles. b) Hypophysectomized alone showed a progressive decrease in body weight and absence of estrous cycles. c) In both groups of hypophysecto-

TABLE I. Effect of Sex of Pituitary Gland Grafted into Hypophysectomized Female Mice on Body Weight and on Estrous Cycles.

Group	No. of mice	Body wt in g (mean) Before operation	1 mo after	2 mo after	Presence of estrous cycles*
Sham operated controls	15	16.50 ± .38	19.25 ± .39	22.90 ± .42	Yes 15/15
Hypophysect. bearing ♀ pituitaries	15	15.91 ± .41	17.62 ± .44	18.94 ± .39	" 15/15
Hypophysect. bearing ♂ pituitaries	22	16.20 ± .36	17.90 ± .34	19.05 ± .28	" 22/22
Hypophysectomized	12	16.80 ± .45	15.10 ± .50	15.00 ± .49	No 12/12

* Normal estrous cycles in animals bearing pituitary grafts appeared at 10 to 15 days following operation.

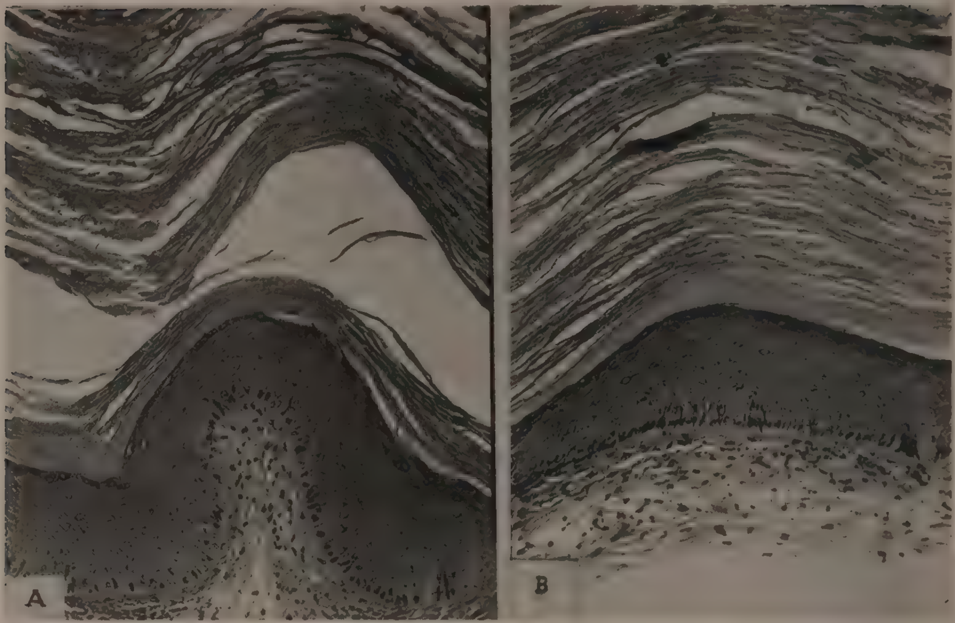

FIG. 1. Histological sections taken from vaginal grafts in castrated male mice with grafted ovaries, bearing either a female (A) or a male (B) hypophysis.

mized females bearing either a female or male hypophyseal gland, progressive increase in body weight and normal estrous cycles were observed.

Table II shows the results of the second experiment. Both groups of castrated males with grafted ovaries showed the typical noncycling vaginal reaction characteristic of a continuous estrogenization, regardless of the sex of the pituitary gland grafted (Fig. 1).

Discussion. From the results described, it seems clear that hypophysectomized female mice bearing either female or male pituitaries had normal estrous cycles. These results agree with those obtained by Harris and Jacobson(3,4) in the rat. Furthermore, castrated male mice with grafted ovaries showed a continuous noncyclic vaginal stimulation regardless of the sex (female or male) of the hypophyseal tissue grafted.

TABLE II. Effect of Sex of Pituitary Gland on Development of Vaginal Grafts in Castrated Males with Grafted Ovaries.

Group	Pituitary	No. of mice	Histology of vaginal grafts
Castrated with grafted ovaries	♂ (own)	10	Cornified noncyclic 10/10
Idem	♀	8	*Idem*
"	None	2	Atrophic or reabsorbed

These results would imply that the normal cyclic function of the female pituitary as opposed to the non-cyclic of the male, as it was formerly described, would not reside in a sex difference of the hypophyseal tissue in itself, for in our experiments, the property of cycling or non-cycling seemed to be determined by the sex of the host, rather than that of the pituitary gland they carried.

In the light of these data it is rather difficult to decide as to where and how the sex difference in gonadal function of the hypophysis originates. Not being involved, as it appears, the sex of the hypophyseal gland, and since anatomically this organ is related to the hypothalamus in the brain through vascular and nervous connections, it would be perhaps justified to assume that the cyclic or non-cyclic character of the female or male gonadotrophic function might depend upon a sex difference of that nervous structure.

Summary. Estrous behaviour in hypophysectomized female and male mice bearing hypophyseal grafts has been studied. The results were: (1) Hypophysectomized female mice bearing either female or male hypophyseal grafts, showed complete and normal estrous cycles. (2) Castrated males bearing ovarian and vaginal grafts showed a continuous noncyclic type of vaginal stimulation regardless of whether they had been grafted with male or female pituitaries. (3) The characteristic cyclic function of the female hypophysis may be related to the cyclic function of some other controlling structure rather than being dependent upon the pituitary gland itself.

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Threshold of the Enterogastric Reflex.* (22309)

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Introduction of acid into the duodenum causes a prompt and complete inhibition of gastric motility. This phenomenon has been named by Thomas, *et al.*, the "enterogastric reflex"(1). In a previous study, we reported this inhibition in the human subject and found that it occurred in patients with vagotomy, extensive sympathectomy, partial gastric resection, and spinal cord transection(2). It is the purpose of the present study to explore the threshold of this inhibitory mechanism and to determine its presence in patients with gastric achlorhydria.

Method. As previously described(2), a duodenal tube was passed under fluoroscopic control. A second tube with an attached balloon was passed into the antral portion of the stomach, and gastric motility was recorded. Studies were performed on 11 normal human subjects, varying both the volume and concentration of hydrochloric acid solutions instilled via the duodenal tube. In most patients, 2 or more determinations were made during the same study period—a total of 25 studies. In addition, the same study was performed on 3 patients with persistent gastric achlorhydria as determined by histamine stimulation.

Results. 1. *0.075 N HCl*: In 4 patients, 100 cc of 0.075 N HCl introduced into the duodenum caused a prompt inhibition of gastric motility varying from 26 to 39 minutes. Twenty-five cubic centimeters of the same solution in one patient caused only a 10 minute period of inhibition. 2. *0.05 N HCl*: One hundred cubic centimeters of 0.05 N HCl infused into the duodenum of 5 subjects resulted in cessation of gastric motor activity varying from 9 to 26 minutes. In 2 patients, 25 cc of the same solution instilled into the duodenum caused a 5 and a 6 minute inhibition, respectively. 3. *0.025 N HCl*: One hundred cubic centimeters of 0.025 N HCl was introduced into the duodenum of 3 subjects and was immediately followed by inhibition of gastric motility lasting for 9 to 16 minutes. In 1 patient, 25 cc of this solution produced a 10-minute period of inhibi-

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tion. 4. *0.01 N HCl*: One hundred cubic centimeters of 0.01 N HCl was instilled into the duodenum of 3 patients and resulted in a gastric motor inhibition lasting 5 to 8 minutes. In 1 patient, 50 cc of this solution inhibited gastric activity for 5 minutes; in 2 patients, 25 cc of the same solution inhibited gastric contractions for only 3 minutes.

In the 3 patients with persistent histamine achlorhydria, 0.1 N HCl instilled into the duodenum resulted in prompt and profound inhibition of gastric motility. It was indistinguishable in latent period, degree, and duration from that occurring in subjects with normal gastric secretion.

Conclusions. 1) Duration of inhibition of gastric motility varies directly with volume and concentration of HCl solutions introduced into the duodenum. 0.075 N HCl produces definitely shorter inhibition than was previously found with equal volumes of 0.1 N HCl. When concentration of HCl is reduced to 0.01 N and volume reduced to 25 cc, the inhibitory period is very brief, lasting only 3 minutes. 2. In 3 patients with complete histamine achlorhydria, the inhibitory effect of intraduodenal acid on gastric motility was the same as that in individuals with normal gastric secretion.

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Organ-Specific Effects of Cortisone on Protein Synthesis in Protein Depleted Rats.* (22310)

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The mechanism of action of cortisone and other adrenocortical steroids on protein metabolism has been the subject of many investigations. The theories and observations of some investigators(1-3) seem to favor a primary anti-anabolic effect while other workers(4-6) favor a primary catabolic mechanism. Still other reports(7) support a more complex interpretation.

Previous work(8) indicated that rats maintained on a completely protein-free diet offered a unique situation in which to study the effects of cortisone. The present communication is a preliminary report of results obtained from a study of the incorporation *in vitro* of glycine-2-C^{14} into protein fractions of liver slices and intact isolated diaphragms from protein depleted rats treated with cortisone. The liver and diaphragm were studied in the hope that they might show differences in their ability to incorporate labeled amino acids and that these changes would reflect the influence of cortisone on protein synthesis or breakdown.

Material and methods. Young male rats were maintained on 3 experimental regimens (8). These regimens included: 1) normal synthetic diet; 2) protein-free synthetic diet; 3) protein-free synthetic diet plus daily subcutaneous injections of 2.5 mg of cortisone 100 g body weight. All groups were maintained on the experimental regimens for 12 to 17 days. Following the period of treatment the rats were lightly anesthetized with ether and sacrificed by exsanguination from the heart. The livers and diaphragms were removed, weighed, and placed immediately in chilled Krebs-Henseleit media equilibrated with 95% oxygen and 5% CO_2. The incu-

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TABLE I. *In Vitro* Incorporation of Glycine-2-C^{14} into Protein Fractions of Rat Liver Slices and Intact Isolated Diaphragms. (Values are expressed as the mean of the individual values ± the standard error of the mean.)

	Incorporation of glycine-2-C^{14} into LIVER protein fractions		Incorporation of glycine-2-C^{14} into DIAPHRAGM protein fractions	
Treatment	Without added non-labeled amino acids (cpm/mg protein)	With added non-labeled amino acids[*] (cpm/mg protein)	Without added non-labeled amino acids (cpm/mg protein)	With added non-labeled amino acids[*] (cpm/mg protein)
Normal controls	260 ± 37.2 (4)[†]	No observations	38 ± 5.7 (3)	36 — (1)
Protein-free diet controls	128 ± 9.0 (7)	201 ± 11.7 (5)	41 ± 5.6 (7)	60 ± 10.7 (5)
Protein-free diet + cortisone	164 ± 7.0 (7)	149 ± 11.9 (5)	61 ± 3.8 (7)	82 ± 6.6 (5)

[*] Final concentration of L-isomer, 10 $\mu g/ml$.

[†] The No. of animals in which duplicate observations were made are shown in parentheses.

bation and isolation of the protein fraction was essentially as described by Krahl(9) except for minor technical changes. Approximately 400 mg of liver slices or one hemidiaphragm was placed in screw-cap incubation bottles. These bottles contained Krebs-Hensleit media (liver, 2 ml; diaphragm, 1 ml) and glycine-2-C^{14}, 11.6 $\mu g/ml$ (specific activity, 1.25 mc/mM). A mixture of non-labeled amino acids was also added to some of the flasks in addition to glycine-2-C^{14}. This mixture contained l-cysteine, dl-lysine, l-histidine, dl-leucine, dl-threonine, dl-phenylalanine, dl-valine, l-arginine, dl-methionine, dl-isoleucine, and dl-tryptophane and was adjusted to pH 7.0 with $NaHCO_3$. The tissues were incubated with shaking for 2 hours at 37°C. Radioactivity in protein fractions was determined in an internal gas flow counter. All samples were counted at maximum self-absorption. Protein content was determined by the method of Lowry *et al.*(10).

Results. In confirmation of our earlier findings(8) the animals on the normal synthetic diet gained weight, those on the protein-free diet lost about 20% of their initial body weight, and those on the protein-free diet plus cortisone lost 40% of their starting weight ($P<0.001$). Liver weight/body weight ratios were significantly increased ($P<0.001$) in the animals receiving the protein-free diet plus cortisone. The protein-free diet caused a 30% decrease in the mean diaphragm weight when compared to normal values ($P<0.001$). The increase in liver weight in the cortisone treated animals was due in part to an increased protein content. Whereas cortisone treatment caused no change in the diaphragm weight/body weight ratio the liver weight/body weight ratio increased. Thus while the diaphragm suffered with other tissues from the wasting effects of cortisone, the liver was spared.

Liver slices from animals on a protein-free diet showed a decreased utilization of glycine-2-C^{14} compared with that of liver from normal controls ($P = 0.01$) (Table I). Krahl (9) observed a similar effect in fasted rats. It was possible to increase utilization of glycine-2-C^{14} by the addition of a mixture of essential non-labeled amino acids ($P<0.001$). Liver slices from animals on a protein-free diet plus cortisone showed an increased utilization of glycine-2-C^{14} compared with that of liver from protein-free dieted animals ($P = 0.01$); in this case the addition of other amino acids had no stimulatory effect.

In the diaphragms, the diet had no effect on the utilization of glycine-2-C^{14} (Table I). Treatment with cortisone caused an increased utilization of glycine-2-C^{14} by the diaphragms ($P = 0.01$). Addition of non-labeled amino acids to the diaphragms from the cortisone treated animals caused a still further increased utilization of glycine-2-C^{14} ($P<0.02$).

Summary and conclusion. In these experiments cortisone had no antisynthetic effect; its effect on protein synthetic reactions was to increase synthesis in the diaphragm. Since liver is spared and diaphragm is not spared against the wasting effects of cortisone, the chief effect of cortisone according to our observations must be its influence on catabolic reactions. Thus the organ-specific effects of cortisone can be explained on the basis of increased catabolism in diaphragm but not in liver.

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Effect of Serotonin on Renal Excretion of Sodium; Possible Relation to Rauwolfia Action.* (22311)

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The administration of reserpine has been found to be associated with a decrease in the concentration of serotonin in brain, platelets and intestines(1). It has also been demonstrated that rauwolfia alkaloids can produce occasional salt and water retention even to the point of congestive failure(2). The present study was undertaken to investigate the possibility that this effect of rauwolfia might be mediated through serotonin.

Materials and methods. Forty convalescent patients free of cardiovascular, renal, metabolic or endocrine disease were selected for the study. All but 3 were bed patients, and all were afebrile and on a regular diet. All were given an infusion of 250 ml of 5% glucose and water. One or 2 mg of serotonin base‡ were added to the infusion of 18 subjects, the remaining 22 serving as controls. Those receiving serotonin were unaware that any drug had been added to their infusion, and did not experience any blood pressure change or discernible local or systemic reaction. Comparable conditions and identical procedures were employed in both groups. Each subject emptied his bladder as completely as possible in the sitting or standing position. One hour after breakfast the patients voided and then drank 200 ml of water. Exactly one hour later, they again voided and were immediately given the infusion which was administered at a constant rate to require one hour for its completion. Urine specimens were obtained at the close of the infusion and at exactly hourly intervals for 2 additional hours. All hourly urine specimens, beginning with those obtained at the start of the infusion, were measured and analyzed for sodium, potassium (by flame photometer), and chloride. For the purposes of this study, the excretion rates in the hour's specimen obtained at the end of the infusion were compared with excretion rates per hour in the combined 2 hourly specimens subsequently collected.

Results. The mean excretion of sodium in mEq/hr in the control group was 7.7 at the start of the infusion, rose to 9.4 at its close, with a further increase to 9.6 in the 2-hour period after the infusion. In the serotonin group the initial value was 5.9, rose to 7.2 at

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† National Heart Institute trainee.

‡ Serotonin creatinine sulfate was supplied by Dr. E. W. Young of Upjohn Co., Kalamazoo, Mich.

TABLE I. Changes in Urine Volume and Electrolyte Excretion Rates following Infusion with and without Addition of Serotonin.

	Control group					Serotonin group			
		Na	K	Cl			Na	K	Cl
	Vol, ml/hr	mEq/hr				Vol, ml/hr	mEq/hr		
1.	57 - 68†	4.4 - 5.7	3.4 - 3.4	5.6 - 5.7	1.*	189 - 75	13.6 - 3.2	7.8 - 3.4	19.6 - 6.3
2.	168 - 145	2.6 - 4.9	4.9 - 6.8	4.9 - 6.6	2.*	109 - 144	12.7 - 11.1	5.3 - 4.4	14.3 - 13.4
3.	110 - 30	3.3 - 1.4	1.3 - 1.3	3.4 - 1.7	3.*	186 - 96	4.3 - 1.8	2.8 - 1.5	5.9 - 2.7
4.	100 - 118	1.9 - 8.9	.7 - 1.8	1.8 - 7.3	4.*	62 - 155	9.1 - 6.8	1.8 - 3.6	8.8 - 8.5
5.	78 - 76	3.0 - 3.3	3.4 - 2.9	4.7 - 3.8	5.*	59 - 102	3.5 - 3.4	2.7 - 3.7	5.5 - 5.9
6.	40 - 28	3.2 - 1.9	2.1 - 1.6	3.8 - 2.3	6.	47 - 31	5.2 - 2.6	3.7 - 3.3	9.6 - 5.8
7.	23 - 34	1.5 - 4.2	2.0 - 3.3	3.8 - 7.0	7.	36 - 45	5.8 - 5.4	2.2 - 3.5	6.1 - 6.5
8.	59 - 40	11.3 - 6.8	6.0 - 2.6	11.0 - 6.1	8.	77 - 114	5.8 - 4.6	2.0 - 1.9	5.1 - 3.7
9.	86 - 136	9.7 - 9.7	3.0 - 3.5	9.7 - 10.5	9.	57 - 89	2.4 - 3.6	1.3 - 1.5	3.0 - 4.3
10.	129 - 134	4.4 - 5.4	2.9 - 2.7	5.4 - 5.4	10.	83 - 73	6.3 - 10.2	5.3 - 4.2	9.5 - 9.2
11.	94 - 205	9.6 - 9.4	4.0 - 3.8	10.8 - 9.5	11.	208 - 185	6.2 - 4.4	5.6 - 9.4	6.2 - 3.9
12.	184 - 108	4.5 - 3.3	2.7 - 4.2	3.4 - 3.7	12.	120 - 106	4.3 - 3.6	2.4 - 3.9	4.3 - 6.0
13.	53 - 48	7.5 - 6.7	4.3 - 4.4	9.5 - 8.5	13.	540 - 75	11.6 - 1.9	3.6 - 2.6	4.9 - 2.2
14.	150 - 140	10.6 - 12.6	4.6 - 5.7	14.1 - 16.7	14.	136 - 93	7.8 - 7.3	4.5 - 6.2	9.7 - 10.4
15.	181 - 111	10.2 - 10.6	8.2 - 5.5	13.0 - 12.9	15.	144 - 134	19.5 - 13.5	6.8 - 6.7	23.2 - 17.1
16.	89 - 174	6.3 - 15.5	.9 - 2.9	6.3 - 16.9	16.	47 - 58	5.8 - 3.9	5.3 - 4.9	9.1 - 6.8
17.	60 - 46	11.1 - 8.1	5.5 - 4.7	15.0 - 11.7	17.	41 - 134	.9 - 5.7	.7 - 5.5	.9 - 7.2
18.	414 - 129	17.5 - 15.2	3.7 - 3.2	15.9 - 17.0	18.	178 - 175	4.7 - 2.7	8.6 - 2.6	8.3 - 2.4
19.	144 - 146	2.9 - 1.2	2.8 - 3.1	3.2 - .8					
20.	216 - 181	.8 - 1.1	4.6 - 4.6	2.3 - 2.7					
21.	154 - 105	41.0 - 29.7	5.9 - 5.7	42.3 - 32.0					
22.	270 - 310	40.0 - 45.8	6.5 - 7.6	39.5 - 45.0					
Avg	130 - 114	9.4 - 9.6	3.8 - 3.9	10.8 - 10.6	Avg	129 - 105	7.2 - 5.3	4.0 - 4.0	8.6 - 6.8

* These received 1 mg serotonin base, the remainder 2 mg.

† First figure of each column denotes values obtained at end of infusion; second figure indicates values of the combined subsequent two hourly specimens.

the close of the infusion, then fell to 5.3 in the 2-hour subsequent period. The urine volumes and the rates of excretion of sodium, potassium and chloride as affected by the infusion are shown in Table I. Whereas the urine sodium excretion rate diminished in 10 of the 22 control subjects following the infusion, it decreased in 15 of the 18 patients after the infusion with serotonin. In general, the chloride excretion rate was similar to that of sodium. No consistent trends were noted in either water excretion or urine potassium values.

The concentrations of electrolytes can be calculated readily from these data. The urine sodium concentration rose in 15 of the 22 control subjects after the infusion, but decreased in 15 of the 18 patients after serotonin. The average urine sodium concentration of the control group was 82 mEq/L. in the specimens obtained at the end of the infusion, and rose to 90 mEq/L. in those collected during the subsequent 2-hour period. In contrast, the average values of the serotonin group fell from 74 to 56 mEq/L.

These differences, employing averages as well as geometric means, were determined to be statistically significant ($p = <0.02$).

Discussion. These data indicate that the administration of small doses of serotonin creatinine sulfate may be associated with decreases in renal sodium excretion, occurring principally after an hour's infusion with this chemical. The conditions of the study were such that the changes noted could not be explained by variation in activity, diet or in the adequacy of urine collections as these were comparable for both groups. The mechanism, significance, and the effects of high dosage and prolonged administration remain to be determined. These observations support the view that electrolyte changes following rauwolfia administration may be related to serotonin activity. They also raise the question that serotonin may play a role in the physiologic regulation of sodium metabolism.

Summary. The administration of 1-2 mg of serotonin creatinine sulfate may be associated with significant sodium retention. Electrolyte changes following rauwolfia ad-

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Separation of Antibodies in Syphilitic Rabbit Sera by Electrophoresis-Convection.[*] (22312)

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The method of electrophoresis-convection has been used by Cann *et al.*(1-3) to obtain separations of a number of different types of antibodies. Their work suggested that it might be possible to separate the Wassermann reagin antibody from the antibody producing immobilization of spirochetes in the *Treponema pallidum* immobilization (TPI) test(4) and to relate these antibodies to electrophoretic serum components.

In this study a pool of syphilitic rabbit sera was fractionated by electrophoresis-convection and the resulting materials tested for the Wassermann reagin antibody by the VDRL flocculation test(5), tested for immobilizing antibodies in the TPI test and also tested for agglutinating activity(6). The fractions obtained were characterized by electrophoretic analysis.

Methods. The electrophoresis-convection apparatus used was that described by Raymond(7)[‡]. The operation of the apparatus was carried out in a coldroom at ca. 4°C. A 4 l bottle filled with buffer was placed in the buffer circulating system to increase the volume of circulating buffer to ca. 8 l. The increased buffer volume seemed desirable to minimize pH changes in the buffer during the course of the run. Protein concentrations were estimated by semimicro Kjeldahl nitrogen determinations and the factor 6.25 was used to convert nitrogen to protein. The relative composition of the serum fractions was determined by paper electrophoresis using the method developed by Grassmann and Hannig(8)[§]. The mobilities were determined by moving boundary electrophoresis using the Perkin-Elmer Model 38 Electrophoresis Apparatus. The determinations were carried out at a protein concentration of 0.3% in barbital buffer (pH 8.6, $\mu = 0.1$). The samples were simultaneously dialyzed for 48 to 72 hours against the same 2 l volume of barbital buffer. The specific conductance of the buffer after dialysis was used in the mobility calculations. The mobilities reported are the average of those calculated from photographs of the descending boundaries taken by the Longsworth scanning procedure, at 1, 1.5 and 2.0 hours. The VDRL slide flocculation test was used to measure the relative Wassermann reagin antibody content of the fractions. The serum fractions were not heat inactivated prior to testing. The TPI test with modifications(6) was carried out on serum fractions that had been diluted with normal rabbit serum (Pool I) to a final concentration of 2.0% of serum fraction protein and then heat inactivated at 56°C for 30 minutes. The samples tested were of the order of half serum

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[‡] Manufactured by E. C. Apparatus Co., New York City.

[§] The paper electrophoresis apparatus and densitometer were obtained from E. Miltenberg Inc., New York City.

TABLE I. Serologic Analysis.

	TPI		Agglutination		VDRL			
	Protein conc., %	Titer*	Protein conc., %	Titer‡	Protein conc., %	Undiluted	1:2	1:4
Pool VI (syphilitic) undiluted	6.8	1: 940	6.77	1: 80	6.8	2+	1+	neg
Pool VI 2%	2.0	1: 340	2.0	1: 20	2.0	±	neg	"
pH 7.5 Top	2.0§	1: 820	2.0§	1: 80	5.9	4+	3+	"
7.0 "	2.0§	1: 1170	2.0§	1:160	2.8^5	2+	neg	"
6.5 "	2.0§	1: 1300	2.0§	1: 80	3.4	±	"	"
6.0 "	2.0§	1:10000	2.0§	"	3.4	±	"	"
5.5 "	1.0§	1: 1920†	1.0§	" †	1.8^5	neg	"	"
Pool I (normal) undiluted	6.2	neg‖	6.22	undil. 1+	6.2	"	"	"

* Dilution of serum protein to produce 50% immobilization of spirochetes.
† Titer on basis of a 2.0% solution.
‡ Dilution of serum protein to produce a 2-3 agglutination of spirochetes.
§ Serum fraction protein concentration.
‖ Negative.

fraction protein and half normal rabbit serum protein. All the TPI titers were obtained from a single assay of high sensitivity and are reported as the dilution of the sample necessary to produce 50% specific immobilization of the spirochetes. The same samples prepared for the TPI assay were tested for *T. pallidum* agglutination activity by the method of McLeod and Magnuson(6). The titers reported for comparison were obtained from a single test and are the greatest dilutions of the serum fractions that produced 2 to 3+ spirochete agglutination.

Experimental. The syphilitic serum used in this study was an aliquot of a serum pool from 105 rabbits that had had syphilis for 4½ to 7½ months. They had been inoculated intratesticularly with the Nichols pathogenic strain of *T. pallidum*. The pooled serum and the serum fractions were refrigerated at −10 to −20°C except when fractionated or tested. A total volume of 135 ml of the syphilitic rabbit serum was processed in three batches of 45 ml each. The serum was diluted with an equal volume of buffer which gave a final protein concentration of 3.4%. Fresh sodium phosphate buffer was used for each run. The sample was dialyzed against the buffer in which it was to be fractionated. During each fractionation a current of 0.1 ampere and 8 to 10 volts (field strength ca. 2 volts cm^{-1}) was passed through the system for 94 to 96 hours. Fractionations were carried out in sodium phosphate buffers of 0.1 ionic strength and at a series of pH's 7.5, 7.0, 6.5, 6.0, and 5.5. The first run was made at pH 7.5 and the "bottom" from this run was then fractionated at pH 7.0. The process was repeated through the 5 different buffers. The corresponding portions from the fractionation of each of the 45 ml aliquots were pooled before proceeding to the fractionation at the next pH. At the end of this procedure there were 5 "top" fractions and one "bottom" fraction.

Results. The "bottom," which consisted principally of albumin with a little globulin, gave negative TPI, agglutination and VDRL tests and was discarded. The relative com-

TABLE II. Electrophoretic Analysis.

	Relative composition, %*				Mobilities (cm^2 $volts^{-1}$ sec^{-1})† × 10^5	
	Alb.‡	α-glob.	β-glob.	γ-glob.	β-glob.	γ-glob.
Pool VI	60 (67)	9 (11)	14 (10)	17 (12)		
pH 7.5 top	2	0	4	94		-1.2^0
7.0 "	0	0	11	89		-1.4^2
6.5 "	0	0	10	90	-3.5^2	-1.6^3
6.0 "	4	0	20	76	−3.6	-1.7^5
5.5 "	8	6	30	56	-3.5^3	−1.9

* Relative protein concentrations were estimated by paper electrophoresis except those values for Pool VI in parenthesis which were estimated by moving boundary electrophoresis (protein 1.7%, barbital buffer pH 8.6, $\mu = 0.1$).

† Mobilities were determined by moving boundary electrophoresis (protein 0.3%, barbital buffer pH 8.6, $\mu = 0.1$).

‡ Alb. = albumin; glob. = globulin.

position of the proteins in the 5 "top" fractions was determined by paper electrophoresis. It was found that they contained from 20 to 40% albumin. This indicated a poorer separation than had been previously reported for a similar electrophoresis-convection fractionation of rabbit serum(3). Serologic tests of the fractions showed little if any separation of antibody activities. Therefore each of the 5 "tops" was refractionated in the electrophoresis-convection apparatus using a buffer of the same composition as that in which it was originally separated, The 5 "bottom" fractions were discarded and the "top" fractions were concentrated by pervaporation, dialyzed against pH 7.0 phosphate buffer and the serologic tests carried out. In each of the tests a sample of the unfractionated serum, designated Pool VI; was run at the same protein concentration as the fractions being tested. The serologic results are shown in Table I. The relative composition of the serum fractions was determined by paper electrophoresis and the mobilities determined by moving boundary electrophoresis. The results of the electrophoretic analysis are presented in Table II.

Discussion. The electrophoretic results are in accord with those obtained previously by a similar fractionation of rabbit serum(3). There is a progressive increase in the mobility of the γ-globulin isolated at successively lower pH's and there is no detectable difference in the mobilities of the β-globulins obtained.

The VDRL test results indicate a relative concentration of the Wassermann reagin antibody in the fractions isolated at pH 7.5 and 7.0 and it is probable therefore that this antibody is associated with the slow γ-globulin. There is a relative concentration of the *T. pallidum* immobilizing activity in the fraction isolated at pH 6.0. The difference in titer between the pH 6.0 fraction and the fractions next higher in immobilizing activity corresponds to 2½ to 3 tubes of 2-fold dilution and is believed to be significant. The most reasonable interpretation is to ascribe the immobilizing activity to components in the fast γ-globulin. The serum protein fractions were obtained by a method that produces separations on the basis of differences in electrophoretic mobility. The possibility exists that the differences in antibody activity measured were due to a fractionation of the α or the β globulin components. There was no evidence obtained for differences in mobilities among the α components or the β components of the various fractions. The more likely interpretation however is to associate the differences in antibody activities that have been demonstrated with the differences in mobilities of the γ-globulin fractions that have been measured.

There does not appear to be any significant concentration of agglutinating activity in any of the fractions. This may be a reflection of the importance of more than one antibody in the agglutination reaction(9).

Summary. An electrophoresis-convection fractionation of a pool of syphilitic rabbit serum has been carried out. Significant differences in the mobilities of the γ-globulin components of these fractions have been demonstrated. No such differences in the mobilities of the β-globulin components were found. TPI, VDRL and agglutination studies were carried out. A comparison of the serologic and electrophoretic data indicates that the Wassermann reagin antibody is concentrated in the slow γ-globulin component and the *T. pallidum* immobilizing antibody is concentrated in the fast γ-globulin component. There is no evidence for the concentration of *T. pallidum* agglutinating activity in any of the fractions.

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Serological Response to Japanese B Encephalitis Vaccine of Children and Horses Immune to St. Louis Virus.* (22313)

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In the spring of 1946 an opportunity was afforded one of us (W. McD. H.) to make serological tests on adult Japanese and American troops given a single injection (1 ml) of Japanese B encephalitis (JBE) mouse brain vaccine, then in current use in American troops. Japanese with elevated neutralizing antibody titers to JBE virus and without detectable complement fixing (C.F.) antibody prior to vaccination responded with elevated C.F. titers within 10 days after injection of a single dose of vaccine(1). This observation was subsequently confirmed by Sabin, Ginder, Matumoto and Schlesinger(2). On the other hand, American troops usually had neither neutralizing nor C.F. antibody responses after such an interval and only about 50% responded with significant neutralizing antibodies after the regular course of 3 injections; also, with rare exceptions, there was no C.F. antibody detectable at any time(1,3). This led us to wonder whether a rapid C.F. antibody response after an injection of JBE vaccine might be a specific means of determining whether an individual found to have JBE neutralizing antibody had been previously infected with JBE virus or whether such antibodies might be the result of an infection with St. Louis encephalitis (SLE), West Nile (WN) or another virus in this closely related group. In this connection, 2 of us (W. McD. H. and W. C. R.), while at the Hooper Foundation, had repeatedly observed in performing sero-diagnostic work for the arthropod-borne viral encephalitides that many humans and horses with SLE virus neutralizing and C.F. antibodies also had elevated serological titers to JBE virus. In some instances the titers to the latter agent were higher than those to SLE virus. Similar diagnostic rises occurred with both viruses. Obviously, if both viruses should be present in the same area this would pose a serious diagnostic problem. Also, as has already occurred in our experience and in that of others, antibodies for several of these viruses have been found in populations of other countries prior to isolation of any virus. Which virus gave rise to the antibodies has not always been obvious. The experiments reported in this paper were undertaken to see whether administration of JBE vaccine would permit of specific serologic differentiation for JBE virus infection.

Materials and methods. All sera after collection were frozen in rubber stoppered Pyrex glass tubes until tested. They were shipped with dry ice, then held at –25°C. All sera in a series from the same host and pertaining to one experiment were tested simultaneously. Neutralization tests were performed both by the intracerebral route (in 3 to 4-week-old mice) and by the intraperitoneal route (in

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TABLE I. Serological Response of Human Encephalitis Cases from Kern County, Calif., during Disease and Convalescence and Finally before and after an Injection of Japanese B Vaccine.

Name	Time relation to onset or vaccination (days)	St. Louis			Japanese B		
		Neutralization			Neutralization		
		Intracer.	Intraper.	C F	Intracer.	Intraper.	C F
			× 1000				
Goodloe	8 P.O.†	(1)* 39 (2)* 120	—	(1) <4 (2) "	(1) 100	—	—
	20 P.O.	(2) 220	—	(1) 64 (2) "	(1) 100	—	—
	27 P.O.	(1) 285	(1) >100	(1) " (2) "	(1) 1,600	—	—
	53 P.O.	(2) 770	(1) 1,000	(2) "	(1) 1,000	—	—
	0 P.V.†	(1) 3,200	(1) 160	(1) 4	(1) <100	(1) 80	(1) <4
	11 P.V.	(1) 10,000	(1) >320	(1) 32	(1) 1,000	(1) 10,000	(1) 8
Leflore	16 P.O.	(1) 32	—	(1) 32	(1) 75	—	—
	42 P.O.	(1) 2,000	>1,000	(1) 128	(1) 1,000	—	—
	140 P.O.	—	—	—	(1) 100	—	—
	0 P.V.	>100	>10	(1) 8	(1) 630	(1) 3,200	(1) <4
	11 P.V.	1,000	>320	(1) 8	(1) 320	(1) 6,300	(1) 8
Bugni	Onset	(1) 155	(1) 10	(1) <4	(1) 150	—	—
	45 P.O.	(1) 1,900	—	(1) 64	(1) 3,200	—	—
	120 P.O.	—	(1) 1,000	—	(1) 320	—	—
	155 P.O.	—	(1) 1,000	—	(1) 1,000	—	—
	0 P.V.	(1) 1,000	(1) >320	(1) 16	320	800	(1) 4
	11 P.V.	(1) 5,000	(1) >320	(1) 32	1,300	32,000	(1) 32

* No. in parentheses represent the date on which tests were performed. In any one section (post onset, or pre or post vaccination) in the same vertical column, all those tests with the same numeral in parentheses were performed at one time.

† P.O. = Post onset; P.V. = Post vaccination.

suckling mice) by the virus dilution method, using undiluted and unheated serum. The virus strains used were: for the intracerebral test, SLE, Webster strain, and JBE, Okinawa strain; and for the intraperitoneal test, SLE, Hubbard strain, and JBE, Nakayama strain. Incubation of serum and virus was for 2 hours at 37°C. All mice were of the CFW strain. Complement fixation tests were done by the method of Casals(4), employing serial dilutions of serum, 2 exact units of complement and incubation overnight at 4°C. The antigens were of the benzene-extracted type, using the method of España and Hammon(5). JBE chick embryo type of formalin inactivated vaccine of suitable potency was obtained from Doctor J. E. Smadel of the Army Medical Service Graduate School. Through the courtesy of Doctor W. A. Longshore of the California State Department of Public Health, 3 children whom we had diagnosed by serological methods in 1949 as cases of St. Louis encephalitis were bled on March 8, 1950, then given 1 ml of vaccine and bled 11 days later. Sera for retest taken during the acute and convalescent phase of their illnesses were still present in our deep freeze. Fourteen horses resident in Kern County were selected on the basis of earlier serological testing and antibody status. Two control horses were selected from a non-endemic area on the basis of an absence of detectable antibodies. Through the kindness of Doctor Ben H. Dean of the California State Department of Public Health the horses were bled, injected with 5 ml of vaccine each, then bled again 10 and 30 days later. Aliquots of serum samples from these horses were tested in 3 different laboratories, California State Department of Public Health (EHL), Hooper Foundation, U. of California (WCR) and University of Pittsburgh (WMH and GES), by a variety of methods. The results in the 3 laboratories were essentially alike, and those reported here are the results of tests performed at the University of Pittsburgh.

Results. 1. *Convalescent Patients.* The results of tests made during illness and convalescence, followed by those just before and after vaccination of the 3 children, are presented in Table I. It will be observed that just before vaccination all had detectable neutralizing and C.F. antibody to SLE virus. Goodloe had essentially no antibody of either type to JBE virus. Leflore had only neutralizing antibody, while Bugni had a detectable level of both types of antibody. Following vaccination all but Leflore showed an increase in JBE neutralizing antibody and all showed significant rises in JBE C.F. antibody. In

TABLE II. Serological Studies on Japanese B and St. Louis Antibody Response of California Horses Vaccinated with Japanese B Vaccine.

	Neutralization tests				C.F. tests	
	St. Louis		Japanese B		St.	
Horse	Intracer.	Intraper.	Intracer.	Intraper.	Louis	Jap B
Red	* 4,000	>50,000	20	1,800	<4§	<4
	† 800	”	130	160,000	32	32
	‡				16	16
Boots	4,000	”	1,000	3,000	<4	<4
	8,000	”	10,000	4,000	8	8
					4	<4
Pat	1,000	320,000	12	<100	<4	<4
	500	6,000,000	100	16,000	8	8
					4	4
King	1,000	500,000	250	6,000	<4	<4
	4,000	6,000,000	16,000	1,000,000	8	4
					<4	<4
Jackass	800	>1,000,000	200	<100	8	<4
	1,600	”	400	84,000	16	16
Black	6,300	”	700	200,000	<4	<4
	6,300	”	700	8,400,000	32	32
Marge	1,600	”	500	500	<4	<4
	16,000	”	2,000	>6,300	8	8
					4	<4
Sally	1,000	200,000	25	250	<4	<4
	5,000	400,000	400	250,000	64	64
					16	16
Boots	800	700,000	40	160	<4	<4
	500	1,600,000	25,000	1,000,000	16	16
					8	8
Apache	5,000	1,000,000	70	100	<4	<4
	1,000	>1,000,000	700	6,000,000	16	16
					8	8
Chief	6,000	>50,000	180	1,300	<4	<4
	6,300	”	4,000	1,000,000	16	16
					<4	8
Thunder	4,000	”	40	50	<4	<4
	4,000	”	800	100,000	32	32
					8	8
Pinto	500	>4,000,000	160	6,300	<4	<4
	2,500	”	16,000	1,000,000	16	16
					8	8
Easter	1	<600	16	<100	<4	<4
	50	”	16	”	8	8
					<4	<4
#1564 Cutter	1	<30	1	<130	<4	<4
	1	”	8	”	<4	<4
					<4	<4
#1556 Cutter	1	100	1	<130	<4	<4
	1	<32	8	”	<4	<4
					<4	<4

* Pre-inoculation bleedings. † 10-day bleeding. ‡ 30-day bleeding. § Reciprocal of dilution giving 2+ or higher complement fixation.

general, rises also occurred in SLE antibodies. However, end-points were not always attained, but in some where titrations were complete, significant rises were not demonstrated.

2. *Horses.* Results of the tests on the horses are presented in Table II. The prevaccination sera of 13 of the 16 horses contained significant neutralizing antibody to SLE virus and most of these 13 also had detectable JBE neutralizing antibody titers. Easter had none detectable by the intracerebral method but the intraperitoneal test was not performed with a small enough amount of virus to eliminate the possibility of a trace of antibody. The last 2 horses (controls) in the Table can be considered negative for neutralizing and C.F. antibodies.

Following vaccination, C.F. antibody titers rose rapidly to both SLE and JBE in all those with previous SLE and/or JBE neutralizing antibody. Higher titers were found after 10 days than after 30. Easter gave this same type of response. However, those unequivocally negative for SLE neutralizing antibody prior to vaccination showed no response. Neutralizing antibody response to JBE in the 10-day interval (not tested after 30 days) also appears to have been very pronounced in most horses originally immune to SLE, and the rise was more conspicuous than that to SLE. Easter and the two with no previous immunity showed no detectable response.

Discussion. Both children and horses (resident in the Western United States) and naturally immune to what we assume must be St. Louis virus responded serologically to a single injection of a killed-virus JBE vaccine. This response was indistinguishable from that of persons immune to JBE. The C.F. antibody response was most dramatic, since nonimmunes fail to develop any antibody of this type, regardless of time or even after repeated injections. If one assumes that the horses and children had not been previously infected with JBE virus, the secondary type response demonstrated by JBE vaccine is not specific for previous JBE infection, and the test does not serve any useful purpose in differentiating between those two viruses. It is, however, in all probability a specific secondary immunologic response to a common antigen present in both JBE and SLE viruses.

It will be recalled, however, that in addition to the rapid rise in C.F. antibody a very striking and rapid rise generally occurred also in JBE neutralizing antibodies. In view of the low response rate (7 to 30%) to even 3 injections of vaccine of this same type then in use in normal American troops(6), one of the factors leading to its discontinuance in military preventive medicine, the response in St. Louis immunes, assumes particular interest. The following questions are raised: (1) Is it possible that the few American adults (troops) who had good serological responses to JBE, *i.e.*, high neutralizing antibody titers and positive C.F. tests, represented those few individuals with immunity to SLE, and (2) could this heterologous, booster phenomenon be used for a practical purpose in immunization?

Since JBE immunization with currently available killed vaccine has not been used recently in military groups because its effectiveness has been questioned, it is possible that another antigenically related virus of a more benign nature might be employed as an infecting agent to give basic primary group immunity. This then could be supplemented with an injection of killed JBE vaccine to obtain the response noted in these experiments. Certain strains of West Nile virus have already been demonstrated to be quite benign(7) and suggest themselves for this purpose.

With this in view, experimental work in laboratory animals has been undertaken by the University of Pittsburgh group and will be the subject of subsequent reports(8).

Summary and conclusions. Three children convalescent from St. Louis encephalitis and a group of 16 horses, 13 of which had naturally acquired antibodies to St. Louis virus, were given one injection with a killed Japanese B encephalitis virus vaccine. After 10 or 11 days all individuals previously infected with St. Louis virus showed complement fixing antibody rises to Japanese B and St. Louis viruses and most also showed neutralizing antibody rises, particularly to Japanese virus. Those horses not previously immune to St. Louis virus showed no response. The response of these St. Louis immune hosts was identical to that shown by persons with naturally acquired immunity to Japanese virus. Normal individuals show no such response even after several injections of vaccine. This immediate, secondary type immunologic response to an injection of vaccine is not specific for Japanese virus but to an antigen common to Japanese and St. Louis viruses and probably to other viruses in this complex. It is suggested that immunity acquired by active infection with one member of the group may serve as good basic or primary immunization for other representatives and a high and probably effective protection response can be stimulated subsequently by giving one "booster" injection of a killed virus vaccine representing an immunologically related virus.

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Immunity of Hamsters to West Nile and Murray Valley Viruses Following Immunization with St. Louis and Japanese B.* (22314)

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In a previous paper(1) it was shown that human convalescents of St. Louis encephalitis (SLE) virus and horses with naturally acquired SLE immunity responded quickly with a secondary type of serological response to Japanese B (JBE) virus after one injection of JBE virus, killed vaccine. This paper deals with animal challenge by the subcutaneous route with West Nile (WN) and Murray Valley encephalitis (MVE) viruses following immunization with JBE and SLE viruses belonging to the same immunologic group. Serological crossing between SLE, JBE, WN, and MVE viruses has been demonstrated by neutralization, complement fixation, and hemagglutination tests by a large number of workers and antigenic overlapping of this group is well recognized. Cross-protection tests using the intracerebral challenge of immunized mice have not shown such crossing or have given inconclusive results with two exceptions as noted below. On the basis of the usual lack of cross-protection in mice has rested the principal method of differentiation of members of this group as distinct viruses. Recently, however, Ruchman reported that he had demonstrated cross-protection between SLE and WN using immune rabbits and guinea pigs as test animals(2). His criterion of demonstrated disease in those animals involving tests with recognized strains of SLE and WN was the development of fever. Cross vaccination studies by Pond, Russ, Rogers, and Smadel(3) have demonstrated the relationship between JBE and MVE, since JBE vaccinated mice were satisfactorily protected against peripheral challenge with MVE virus.

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MVE vaccinated mice, however, were not significantly protected against JBE, although there was a suggestion of slight crossing.

In our work, Syrian hamsters have been found susceptible to high dilutions of MVE virus by peripheral routes of infection. Previously, Smadel and others had shown them to be highly susceptible to WN by these routes(4). Because of the low degree of susceptibility of mice by peripheral routes, we felt that hamsters would be a more suitable laboratory animal for demonstrating cross-resistance between certain viruses of this group, if such existed.

Materials and methods. Viruses used were JBE, Nakayama strain, mouse passage 44; SLE, Webster strain, mouse passage number unknown; MVE, Strain #1, mouse passage 12; and WN, B956, mouse passage 28. Stock virus was prepared as 20% infected mouse brain suspension in 50% normal rabbit serum (inactivated at 56°C for 20 minutes) broth and stored in sealed glass ampoules in a CO_2 ice chest. Infectivity of the virus was determined by intracerebral inoculation of 3-week-old CFW mice with 0.03 ml of serial 10-fold virus dilutions made in 0.2% bovine albumen broth. Hamsters were obtained from the Lakeview Hamster Colony of New Jersey. Hamsters from this breed had been found susceptible to infection with this group of viruses. Animals previously tested from a local breeder had proved resistant to SLE virus by even the intracerebral route. Groups of hamsters were immunized when 6 weeks of age to either JBE or SLE viruses by a series of 3 intraperitoneal injections of live virus given as follows: *JBE*: first injection, 0.1 ml 10^{-2} dilution of virus; 10 days later, 0.1 ml 10^{-1} dilution of virus; 10 days later, 0.2 ml 10^{-1} dilution of virus. Challenge was made 12 days later. *SLE*: first injection, 0.1 ml 10^{-2} dilution of virus; 12 days later, 0.1 ml 10^{-1} dilution of virus; 7 days later, 0.2 ml 10^{-1} dilution of virus. Challenge was made 13 days later. For challenge controls, normal hamsters of the same shipment were set aside at the beginning of the immunization period. The neutralization index of serum was determined by employing serial dilutions of virus mixed with undiluted serum, incubated at 37°C for 2 hours, and inoculated by the intracerebral route into 3-4-week-old mice.

TABLE I. Susceptibility of Hamsters by Route of Inoculation.*

Virus	Log LD_{50} of endpoint dilution		
	Intracer.	Intraper.	Subcut.
Japanese B	7.0	<1.0	—
St. Louis	7.8	<1.0	—
Murray Valley	8.0	8.1	7.0
West Nile	9.5	9.2	8.7

* Inoculum—.1 ml.

Results. 1. The susceptibility of hamsters to these viruses as determined by route of inoculation (*Table I*). The inoculum was 0.1 ml. Both MVE and WN viruses were shown to be highly pathogenic by the peripheral routes tested, while neither JBE nor SLE exhibited this peripheral pathogenicity. The four viruses, JBE, SLE, MVE, and WN were all pathogenic by the intracerebral route.

2. *Immunization and Cross Challenge.* Two groups of hamsters were immunized, one to JBE virus and the other to SLE by the methods described. The results of homologous and cross challenge tests are shown in Table II. Since neither JBE nor SLE were pathogenic by a peripheral route, it was necessary to use the intracerebral route of inoculation for challenge with the homologous viruses. For both MVE and WN viruses (heterologous challenge) the *subcutaneous* route of inoculation was used. It will be observed that animals immunized with JBE virus were solidly immune not only to the homologous virus (JBE) but also to both the MVE and WN viruses (protection index greater than 5 to 7 logs). This experiment was repeated with similar results. Immunization with SLE also resulted in complete protection against challenge with the homologous (SLE) virus, but it may be observed that SLE immunization did not result in complete protection against either MVE or WN viruses but that significant protection, nevertheless, did occur. In the case of both MVE and WN viruses protection was most obvious against the most concentrated virus inoculum. This phenomenon frequently observed after ho-

TABLE II. Results of Virus Challenge of Immunized Hamsters.

Immunizing virus	Challenge virus	Route of challenge	Virus dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Log LD$_{50}$	Log protection index
JBE	JBE	i.c.	0/4*	0/4	0/4	0/4							<1.0	>7.5
None	”	”					4/4	4/4	4/4	3/4	1/4		8.5	
JBE	MVE	s.c.	0/4	0/4	0/4	0/4	0/4	0/4					<1.0	>5.2
None	”	”					4/4	3/4	1/4	0/4			6.2	
JBE	WN	”	1/4	0/4	0/4	0/4	0/4	0/4					<1.0	>7.3
None	”	”						4/4	2/4	4/4	0/4	0/4	8.3	
SLE	SLE	i.c.	0/4	0/4	0/4	0/4	0/4						<1.0	>6.8
None	”	”					4/4	4/4	3/4	4/4	2/4		7.8	
SLE	MVE	s.c.	0/4	1/4	2/4	3/4	3/4	2/4	0/4				?	?
None	”	”				4/4	4/4	2/4	0/4	0/4			6.0	
SLE	WN	”	0/4	1/4	3/4	2/4	2/4	1/4	1/4				?	?
None	”	”					4/4	4/4	3/4	3/4	4/4	0/4	9.3	

* Mortality ratio = No. dying/No. inoculated.

mologous challenge of incompletely immunized animals has been discussed by Schlesinger(5). Because of this zone-like reaction the LD$_{50}$ and the protection index were not calculated.

3. *Antibody Response as Related to Survival.* To determine whether resistance to challenge with any virus correlated with the neutralizing antibody titer for that virus, certain animals prepared for use in the above experiments were sacrificed and bled. This was planned after completion of the challenge of the SLE immunized hamsters, so in this instance 4 hamsters surviving homologous challenge were bled. Four JBE immune hamsters which had not been challenged were sacrificed and bled at the time all the others were challenged. Sera from these 4 animals were pooled. All these sera were tested for neutralizing antibody titer against MVE and WN viruses as well as against the homologous viruses. The results are shown in Table III. It will be noted that in the case of those animals immunized with SLE virus, even though bled after intracerebral challenge with homologous virus, thus receiving more immunization than those that were immunized with JBE virus, the antibody titer to MVE and WN viruses was much lower than in those animals immunized with JBE virus. In a parallel manner, the SLE immunized group were less resistant to challenge inoculation with the two heterologous viruses than were those immunized with JBE.

Discussion. The importance of demonstrating cross-protection amongst this group of viruses, especially by peripheral challenge, is evident when we realize that this route more nearly represents the type of natural exposure expected in an arthropod-borne disease. The possible practical application of such a finding was conceived as a result of earlier work on the antibody response to one injection of JBE virus vaccine of children and horses in California who had had a previous, naturally acquired SLE infection(1). Since it was not practical to challenge either the children or the horses with a peripheral inoculation of JBE to demonstrate whether they were really immune, as suggested by the antibody response, it was necessary to find a suitable small laboratory animal that was susceptible to peripheral challenge. The hamster has served such a purpose for challenge with MVE and WN viruses, and cross immunity to each of these has been shown following immunization with either JBE or SLE virus.

TABLE III. Neutralization Indices of Sera from Immunized Hamsters Used in Challenge Tests.

Immune hamster serum	Virus Homologous JBE	SLE	Heterologous MVE	WN
Japanese B (pool)	2000	—	214	11300
St. Louis				
Serum 1	—	514	<5	650
2	—	163	15	1180
3	—	<163	8	650
4	—	163	10	<206

The development of cross immunity in hamsters, together with the antibody findings with SLE immune children and horses injected with JBE vaccine lend further support to the hypothesis that any one virus in the group (possibly a benign strain of WN) if used as a living immunizing agent, might furnish basic, partial protection against all and could be effectively supplemented by a single injection of a specific killed vaccine for the virus found in the area under consideration. Repeated injections of killed vaccine alone, for JBE, the only member of this group of viruses used extensively for immunization, have been shown to be relatively ineffective in producing a neutralizing or complement fixing antibody response in adult man, and hence of questionable value in affording clinical protection. Further work along these lines, using hamsters and monkeys with other combinations of viruses in this group, is now in progress and will be reported shortly.

Summary and conclusions. An available strain of Syrian hamsters was found to be highly susceptible to WN and MVE viruses by peripheral routes of inoculation. Groups of animals of this species were then immunized with JBE and SLE viruses and subsequently challenged subcutaneously with serial dilutions of WN and MVE viruses. Complete protection against these two agents was afforded by JBE immunization, and protection of a lesser degree obtained by SLE immunization. Neutralizing antibody titers of the immunized hamsters to the 2 heterologous challenge viruses paralleled their peripheral resistance in that the JBE virus immunes had higher neutralizing antibody titers to WN and MVE viruses than did the SLE virus immunes. These findings together with previously reported data support the hypothesis that cross immunity to these viruses probably occurs in man and that there might be some practical application of this concept in the field of human immunization. Further work with animals using other virus combinations is in progress.

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SECTION MEETINGS

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Cytoplasmic Fractionation of Kidney Proteins.* (22315)

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The kidney, like the liver, the heart, and certain other organs, has the capacity to hypertrophy and to atrophy under suitable experimental conditions. When one kidney is removed from the rat, the remaining kidney immediately enlarges, and at the end of 6 days, its protein content has increased by approximately 35%(1). If the rat is starved for a period of 7 days, the kidneys may loose 20% of their structural protein(2). It is not known whether the protein added to or lost from the kidney involves certain cellular structures or whether proteins from all cellular components are equally labile. In order to provide an answer to these questions, the distribution of total protein in the various cytoplasmic fractions of normal, hypertrophied, and atrophied kidneys are determined after homogenization and differential centrifugation,

Methods. Female albino rats of the Slonaker-Addis strain weighing 175-185 g were used in all experiments. Renal hypertrophy was produced by removing right kidney while animal was under ether anesthesia and allow-

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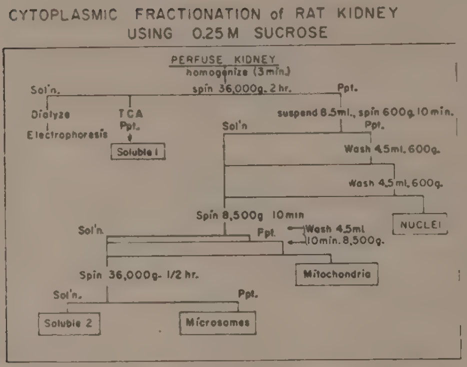
CYTOPLASMIC FRACTIONATION of RAT KIDNEY
USING 0.25 M SUCROSE
PERFUSE KIDNEY
homogenize (3 min.)
spin 36,000g. 2 hr.
Sol'n.
Ppt.
Dialyze
Electrophoresis
TCA
Ppt.
Soluble I
suspend 8.5ml., spin 600g. 10 min.
Sol'n
Ppt.
Wash 4.5ml. 600g.
Wash 4.5ml. 600g.
NUCLEI
Spin 8,500g 10 min
Sol'n.
Ppt.
Wash 4.5ml
10min. 8,500g.
Mitochondria
Spin 36,000g- 1/2 hr.
Sol'n.
Ppt.
Soluble 2
Microsomes

FIG. 1. Flow diagram of fractionation procedure.

ing remaining left kidney to enlarge for 6 days. Renal atrophy was produced by withdrawing all food for 7 days. During this time, the animals subsisted on water only. Rats were anesthetized with ether and their kidneys perfused *in situ* with 0.89% sodium chloride solution as described previously(3). The capsule was removed and the kidneys were blotted dry and transferred to cold room (2°C.) All subsequent steps were carried out at this temperature. Homogenization was carried out in a solution of 0.25 M sucrose containing 0.2% Versene. The homogenate usually contained approximately 3 g of kidney suspended in equal volume of sucrose-Versene solution. The centrifugal method of Hogeboom, Schneider and Pallade(4) was modified slightly as shown in Fig. 1. The particulate fractions were resuspended in distilled water using a pestle designed to fit into the plastic centrifuge tubes. Protein from each fraction was precipitated with 100 ml of 7% trichloracetic acid, washed once with fresh 7% trichloracetic acid, twice with a mixture of equal parts of acetone and ether, once with acetone, and finally once with anhydrous ether. After final centrifugation, the last traces of ether were removed by placing the tubes first in warm water bath and then in oven at 90 C for one hour. The tared tubes containing washed, dried protein were then weighed.

Electrophoresis Procedure. An aliquot of the Soluble 1 fraction was dialysed against boric acid buffer, pH 8.6, for 20 hours at 2°C. Electrophoresis was performed on paper between silicone coated glass plates(5). The paper strips were dried under infra-red lamps and stained with brom phenol blue. The stained paper was cut into strips 0.5 cm wide. The color from each strip was extracted in a solution of 10 parts of concentrated NH_4OH and 90 parts of acetone. Color density of extraction fluid was determined in a Coleman Jr. spectrophotometer at wave length of 610 mμ. The diagrams as shown in Fig. 2 were obtained by plotting per cent absorption on ordinate against distance of migration in centimeters along abscissa.

Results. The results are given in Table I. The data are expressed as means ± standard deviation from the mean. The significance of the differences observed was determined by the method of Fisher(6) for small numbers of observation. P values of <0.01 are taken to indicate a significant difference between the means compared.

It can be seen that the per cent distribution of the total kidney protein in various cytoplasmic fractions studied in normal rats and in starved rats are not significantly different. After 6 days of hypertrophy, the nuclear fraction is slightly, but significantly less, while the mitochondrial fraction is somewhat in-

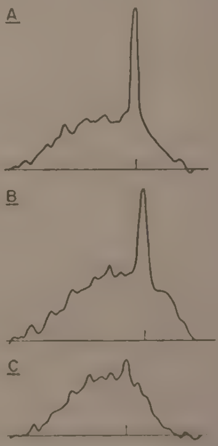

FIG. 2. Electrophoresis diagrams of Soluble 1 fractions. Mobility to the left is toward cathode, mobility to right is toward anode. Arrow indicates point of origin. (A) Normal kidney. (B) Kidney after 6 days hypertrophy. (C) Normal kidney with top fatty layer eliminated.

TABLE I. Percent of Total Protein in Rat Kidney.

Exp. cond. No. exp.	Control 22	Neph. 6 days 8	Starv. 7 days 8	P value
Nuclei	19.3 ± 2.7*	14.9 ± 3.3	20.7 ± 1.5	.01 to .001
Mitochondria	21.5 ± 1.5	23.8 ± 2.3	21.3 ± 1.4	.01 to .001
Microsomes	15.8 ± 1.3	15.5 ± 1.4	15.1 ± 0.6	
Soluble 1	27.8 ± 1.4	28.6 ± 1.4	25.5 ± 0.5	
" 2	16.0 ± 1.6	17.1 ± 2.0	17.5 ± 0.8	
" 1 + 2	43.8 ± 2.0	45.7 ± 2.4	43.0 ± 0.8	

* Stand. dev. $= \pm \sqrt{\frac{\Sigma d^2}{n-1}}$.

creased over what is found in kidneys of normal control rats.

Proteins in Soluble 1 fraction of normal and hypertrophied kidneys were subjected to filter paper electrophoresis as described above. Representative patterns are shown in Fig. 2. When the Soluble 1 fraction was withdrawn with a pipette from below a thin fatty surface layer, most of the peak seen at the origin could be eliminated (Fig. 2C.)

Approximately 14 separate peaks can be identified in the patterns from both normal and hypertrophied kidneys. It has not been possible to distinguish electrophoresis patterns of the Soluble 1 fraction as coming from normal kidneys or from hypertrophied kidneys. Differences exist, but they are small and inconsistent.

Discussion. We have been unable to find reports in the literature dealing with this type of analysis of kidney or other visceral organs undergoing hypertrophy or atrophy. While the flux of tissue protein of liver and kidney during regeneration and atrophy may not be strictly analogous, it will be pertinent to refer to certain experiments performed on liver proteins under these circumstances. Thus, Soroff, Claus and Cohen(7) studied proteins of rat liver by the technic of free electrophoresis. They state that throughout an 8 day period of liver regeneration following 70% partial hepatectomy and throughout a 9 day fast, electrophoretic analysis of soluble rat liver proteins showed no significant difference from those of normal rat liver extracts. de Lamirande, Allard and Cantero(8) report that no difference exists in the soluble proteins of cell fractions from regenerating and normal liver by the technic of electrophoresis. However, a decrease in certain components of the mitochondrial fractions of regenerating liver was observed. Luck(9) found that various fractions of liver protein increase equiproportionally with increased protein intake.

It is clear from the present study that from a loss of 20% of the kidney tissue protein during a fasting period through a gain of approximately 35% during a period of compensatory hypertrophy, the relative protein content of the various cytoplasmic fractions of the kidney remains essentially unchanged. This would indicate that under the experimental conditions outlined above, protein is lost from or added to all cellular fractions of the kidney in a nearly equiproportional manner. The results are inconsistent with the view that a certain fraction of the cellular protein of the kidney is more labile than other fractions under conditions of atrophy and hypertrophy.

Summary. 1. Distribution of kidney proteins in various cytoplasmic fractions has been determined in normal, hypertrophied, and atrophied rat kidney. The Soluble I fraction from normal and hypertrophied kidneys has been subjected to paper electrophoresis. 2. Despite a gain in total protein of approximately 35% in the hypertrophied kidney, and a loss of approximately 20% in total protein in the atrophied kidney, the distribution of proteins in the various cytoplasmic fractions shows only minor deviations from the normal. 3. No consistent differences could be seen in electrophoresis patterns of the Soluble I fraction between normal and hypertrophied kidney. 4. The significance of these findings is discussed.

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Structures Responsible for the Characteristic Purple Color Development in Diphenylamine Reaction with Serum.* (22316)

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Numerous workers(1-4) have reported that the intensity of purple color which resulted when serum was heated with Dische reagent (5) was increased in a variety of diseases. The reaction has been associated with the serum proteins containing carbohydrate moieties: mucoproteins or glycoproteins found in the alpha globulin fraction of serum(2,6,7). Mucoproteins isolated from bovine tonsils and from human urine also gave the color reaction (8,9). The color reaction has been attributed to the presence of sialic acid(10) in the mucoprotein molecule(7). A number of polyhydroxylic acids, neuraminic(11,12), hemataminic(13), and lactaminic(14), have been isolated and appear to be related to sialic acid(15).

It is the purpose of this report to point out that the structure for sialic acid proposed by Gottschalk(16,20) is consistent with structures known to give the characteristic color development when heated with the Dische reagent.

Absorption Studies. Purple color which results when serum or mucoproteins are heated with the reagent is due to 2 absorption regions in the visible spectrum (Fig. 1). One absorption maximum occurs at about 520 mμ while the second occurs at 650 mμ. The absorption maximum at about 520 mμ is the significant one. The second absorbing region at 650 mμ can be attributed to conversion of common hexoses present in mucoproteins to 5-hydroxymethylfurfural when heated in an acid solution(17,18). Absorption maxima in the vicinity of 520 mμ, as will be shown below, can probably be attributed to a deoxyhexose struc-

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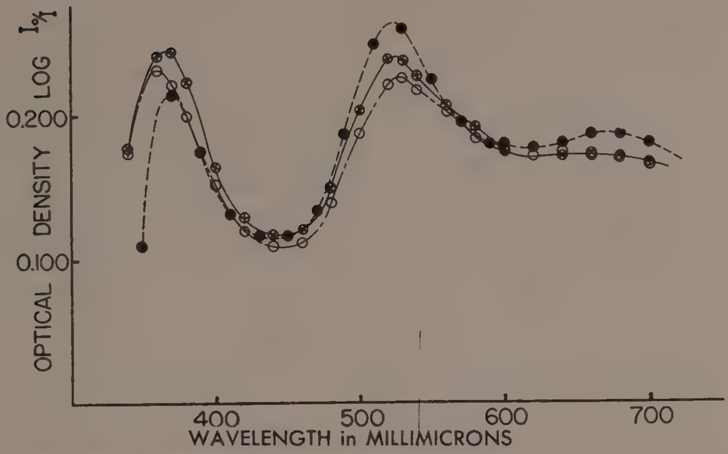

FIG. 1. Absorption curves from diphenylamine reaction obtained with human serum, sialic acid and bovine tonsil mucoprotein. × (circled) Human serum 0.12 ml serum/ml solution according to published procedure(2) using Dische reagent. ○ Bovine tonsil mucoprotein 590 μg protein per ml solution. ● Sialic acid 130 μg/ml solution. To 1 ml solution was added 2 ml of reagent. The mixture heated for 30 min. at 100°, cooled to room temperature and read in a Beckman Model DU Spectrophotometer.

ture which has been postulated(16,20) to be present in sialic and neuraminic acids.

The absorption curves obtained when 40 μg of 2-deoxygalactose§, 40 μg of D-galactal, and 10 μg of fructose, were heated with Dische's diphenylamine reagent may be seen in Fig. 2. In the case of 2-deoxygalactose, and D-galactal, the 40 μg samples were dissolved in 1 ml of water. Two ml of the reagent were added to the aqueous solution and the mixture was heated for 30 minutes in a boiling water bath. Fructose treated in this manner did not develop the characteristic color. When the Dische reagent was added directly to as little as 10 μg of fructose, however, in the absence of water, and the mixture was heated, a pink color developed within a few minutes.‖ The absorption curve obtained with dry fructose heated with the Dische reagent closely resembled the curves obtained from D-galactal and 2-Deoxygalactose as can be seen in Fig. 2. Many substances have been tested with the Dische reagent both in our laboratory and elsewhere(18). Of all the simple substances tested, only the deoxyhexoses and hexals gave the characteristic color development and absorption behavior, when heated in aqueous solution with the unmodified Dische reagent. The ketoses in aqueous solution, when heated with a modified reagent containing a larger fraction of sulfuric acid, also give the characteristic color development. The absorption curves are similar in this case to those obtained when serum, mucoproteins, the hexals and the deoxyhexoses are heated with the Dische reagent.

The absorption curves obtained when sialic acid and a purified mucoprotein were heated with Dische reagent are shown in Fig. 1. Comparison of curves in Fig. 1 and 2 indicate a striking similarity in chromogenic structures present in all substances reacting with the reagent. Acid-catalyzed dehydration of hexoses is well known(19). The conclusion that water is removed from 2-deoxyhexoses and from ketoses under the conditions discussed above, seems justified. The ethylenic ring seen in Fig. 3 thereby becomes a feature common to a dehydrated deoxyhexose, dehydrated fructopyranose, and to hexals. This structure or a common derivative of this

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§ 2-deoxyglucose also shows this characteristic behavior.

‖ Sorbose, galaheptulose, mannoheptulose and sucrose also behave in similar manner.

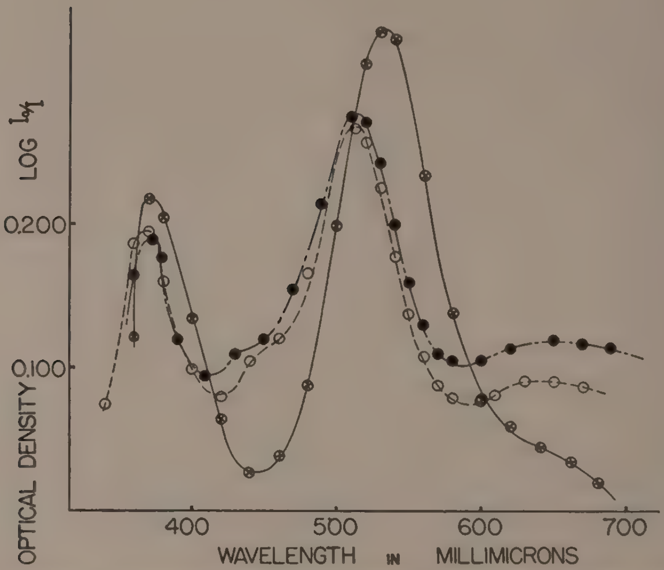

FIG. 2. Absorption curves from diphenylamine reaction obtained with 2-deoxygalactose, D-galactal and fructose. ○ 2-deoxygalactose 40 μg/ml solution. ● D-galactal 40 μg/ml solution. × (circled) Fructose 10 μg. To 1 ml solution was added 2 ml of reagent. The mixture was heated 30 min. at 100°, cooled to room temperature and read in a Beckman Model DU Spectrophotometer. In the case of fructose 3 ml of reagent was added to 10 μg of dry fructose and the mixture heated 30 min. at 100°.

structure accounts for the absorption maxima at approximately 520 mμ. A deoxyketose structure in sialic acid(16,20) would therefore account for the color reaction of this substance with the Dische reagent.

The chemistry of the deoxyhexoses(21) and the hexals(22,23) is also consistent with the viewpoint that a deoxyhexose is present in the sialic acid molecule. A characteristic of the deoxy sugars is their great reactivity and their instability in solutions containing mineral acids. 2-deoxygalactose is almost completely destroyed, with marked discoloration, when heated with 1 N HCl for one minute at 100°. The glycals are stable in alkaline solution, form humins in acid solution and are extremely reactive substances(22,24). The pronounced tendency of sialic acid(25) and of the gangliosides(26) to form humin when heated with acids has been described.

Summary. It has been shown that deoxyhexoses and hexals react with the Dische diphenylamine reagent in a characteristic manner. No other structures except sialic acid and related compounds have been found to react in the same fashion. A deoxyhexose structure for sialic acid has been proposed by Gottschalk. The color given by sialic acid when heated with the reagent is therefore accounted for by the presence of the deoxyhexose. Since sialic acid is considered to be a common constituent of the glycoproteins (mucoproteins), the color given by these substances and serum when heated with the Dische reagent appears to be due to the deoxyhexose present in sialic acid.

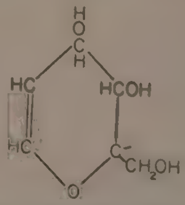

FIG. 3. Ethylenic ring structure common to glycals, and after elimination of a molecule of water, to deoxyhexoses and pyranose form of ketohexoses.

The assistance of Miss Saima Lagg is gratefully acknowledged. Discussions with Dr. G. Blix have been helpful. We are grateful to Dr. A. Gottschalk for calling attention, prior to our seeing the published reports, to the deoxyhexose character of his proposed structures. A number of substances used in this study were obtained through the kindness of several individuals. Dr. T. Reichstein sent generous samples of 2-deoxygalactose, 2-deoxyglucose and D-galactal. The late Dr. G. S. Hudson provided galaheptulose and mannoheptulose. The sample of sialic acid was obtained from Dr. L. Odin.

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Hormonal Requirements of Implantation in the Rabbit.* (22317)

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The phenomenon of implantation in mammals is accomplished by a definite sequence of events which in part are dependent on the endocrine system. It has been adequately demonstrated(1,2,3) that blastocyst growth in the rabbit is controlled by the secretion of the corpus luteum. Partial progestational proliferation in the ovariectomized rabbit could be produced within 5 days by 0.2 mg progesterone daily(4). The injection into rabbits, ovariectomized 20 hours after mating, of progesterone in dosage up to 1.5 mg a day maintained implantation until the 11th day of pregnancy(5). Crude corpus luteum extracts of similar unitage maintained embryos to full term(6). In ovariectomized rabbits, implantation is maintained for 11 days if at least 0.5 mg of progesterone is given twice daily; 1.0 mg given twice daily after the 11th day of pregnancy maintains the embryo to term(7). In the intact non-ovulated rabbit, implantation of transplanted ova requires an initial high dose of progesterone; subsequent treatment prevents the degeneration of the embryo or death of the fetus(8).

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* Thanks are due to Miss Anne P. Merrill and Miss Pauline S. Longo for technical assistance and to Chemical Specialties Co., (Syntex) for progesterone preparations. This investigation was aided-in-part by research grants from the Josiah Macy, Jr. Foundation and the Planned Parenthood Federation of America.

† On leave from Faculty of Agriculture, University of Cairo, Egypt.

Materials and Methods. Sixty-three post-partum and 12 adult virgin rabbits were purchased from Rockland Farms, N. Y. and local dealers and were kept in this laboratory. Two series of experiments were carried out: 1) Eight groups of 3 to 5 animals each were mated twice to fertile bucks and injected intravenously with an ovulating dose of 12 mg of sheep pituitary extract. The does were ovariectomized 24 to 48 hours post coitum and the ovulation points on each ovary were counted. Six groups of does were injected subcutaneously once daily with an oily solution of 0.3, 2 or 25 mg of progesterone 2 or 4 days post coitum. Two other groups were injected subcutaneously once daily with 2 or 25 mg progesterone together with 0.1 mg estradiol. 2) Four groups of 3 does each were mated to a vasectomized buck and injected intravenously with an ovulating dose of sheep pituitary extract. The does were ovariectomized as above; immediately before ovariectomy 24 to 30 fertilized one-day ova were transplanted into the left tubes. The ova were obtained from virgin donors which were superovulated with sheep pituitary extract(9) and mated at the same time as the recipients so that the transplanted ova would be of a corresponding stage with that of the recipient. The experimental does were injected subcutaneously with 2 or 25 mg of progesterone once per day starting either on the day of ovariectomy or the 4th day after mating. In 2 groups 0.01 mg of estradiol was injected once daily. All does were sacrificed on the 10th day after mating; the uteri were examined to determine the number of implantation sites, the diameter of uterine swellings and the weight and length of the fetus. In cases where implantation did not occur, the uteri were flushed with saline and examined under the dissecting microscope. Two control experiments were carried out: (a) Twelve does were mated to fertile bucks, and sacrificed on the 10th day after mating. The number of implantation sites in relation to the number of corpora lutea were counted and the average diameter of uterine swellings, weight and length of the fetus were recorded. (b) Six does were mated to fertile bucks, and were injected subcutaneously once daily with 2 or 25 mg of progesterone. These were sacrificed 15 days after mating and the uteri examined.

Results. In the ovariectomized animals the number of ovulation points on both ovaries ranged from 5 to 18 with an average of 10.9. Implantation occurred in all the animals when the progesterone dose was 2 or 25 mg (Table I). The number of implantations in both uteri ranged from 1 to 3. The number of implantations expressed as a percentage to the total number of ovulation points ranged from 5% to 33% with an over-all average (of 6 experimental groups) of 19.21%. There was no difference in the percentage of implantations between the 2 or the 25 mg groups nor between the groups whose initial injection was on the 2nd or 4th day after mating. Im-

TABLE I. Effect of Progesterone on Implantation and Development in Ovariectomized Rabbits.

Hormones inj. (mg/day)	Start of inj. post coitum (days)	No. of does	Avg No. of ovulations in both ovaries	% of implantations		% of viable embryos		% of ova (or blastocyst) recovered by flushing	
				Range	Mean	Range	Mean	Range	Mean
.3 progesterone	2	3	12.3	0	0			50–73	53
	4	4	7.7	0	0			41–69	52
2 progesterone	2	4	13.3	11–21	19.00	33–50	38		
	4	3	11.3	15–20	17.66	33–66	50		
25 progesterone	2	5	10.4	12–33	23.33	20–80	35		
	4	4	10.2	9–20	15.33	33–66	47		
2 progesterone + .1 estradiol	2	5	7.2	0	0				
25 progesterone + .1 estradiol	2	5	10.1	0	0				

TABLE II. Effect of Progesterone and Estradiol on Implantation of Transplanted Ova in Ovariectomized Rabbits.

Hormones inj., mg/day			Left side after sterile mating				
P*	E*	Doe No.	No. of ovulation points	No. of transferred eggs	No. of implantations	% of implantations	% of viable embryos
2	—	25	3	27	3	11	33
		26	4	24	4	17	50
		27	5	28	2	7	50
2	.01	28	6	25	3	12	66
		29	5	30	2	7	50
		30	5	30	2	7	50
25	—	31	6	30	2	7	50
		32	7	27	2	7	50
		33	4	27	3	11	100
25	.01	34	5	30	1	3	100
		35	3	30	2	7	50
		36	5	29	2	7	50

* P = Progesterone; E = Estradiol.

plantation failed to occur at a dose of .3 mg progesterone, 2 or 25 mg progesterone in combination with 0.1 mg estradiol. In the group injected with 0.3 mg progesterone daily, 3 to 7 ova (or blastocysts) were recovered from the uterus. In the groups injected with 0.1 mg estradiol with progesterone, no ova (or blastocysts) were recovered from both uteri. A few degenerating ova were recovered from the tubes of 3 animals out of 10.

In the control groups "a" which were mated and sacrificed at 10 days the number of implantations expressed as a percentage of the total number of ovulation points ranged from 71 to 100 with an average of 81 and the percentage of viable embryos ranged from 70 to 100 with an average of 81. Judging by the diameter of the uterine swellings and the developmental stage of the embryos in the ovariectomized animals compared with that in the intact animals, there was no evidence of delayed implantation in the former group.

When the gravid uteri of the ovariectomized animals were dissected 35 to 50% of the embryos were viable. In the control group "b" which were injected with 2 mg of progesterone, the percentage of implantations ranged from 80 to 100 with an average of 93 and the percentage of viable embryos ranged from 67 to 100 with an average of 79. In the control group injected with 25 mg progesterone, the percentage of implantations ranged from 80 to 90 with an average of 82. There were no significant differences between these 2 control groups "b". Also the injection of 2 or 25 mg of progesterone in the intact animals did not exert an effect on the percentage of implantation.

In the second series of experiments where fertilized ova were transplanted in the left horn of ovariectomized animals, the number of implantation sites ranged from 1 to 4 and the percentage of implantations ranged from 3 to 17 (Table II). It was noted that the number of implantations was not corresponding to the number of transferred ova. In fact the number of implantations in the one horn was equivalent to those of 2 horn in the previous experiment.

Discussion. There is general agreement that the presence of the luteal hormone is essential for successful implantation but the mechanism by which it makes implantation possible is not fully known. Results of the present experiment suggest that the hormonal requirement for embryonic development is not identical to that required for implantation. The 2 days following implantation seem to be the most critical during the embryonic life (10). It is possible that substances other than progesterone such as androgens or estrogens are required in certain forms or quantities to ensure normal development. The dose of 2 or 25 mg of exogenous progesterone in the ovariectomized rabbits is sufficient to establish implantation but not for further development in some of the cases. Courrier and Kehl (11) and Allen and Heckel(12) have determined that while in the early stages about 1 mg of progesterone is sufficient, in later stages up to 5 mg daily is necessary. Meanwhile ovarian hormones are completely unnecessary in experimental extrauterine pregnancy(13,14).

The absence of implantation when 0.3 mg progesterone was injected daily is either due to incomplete progestational conditions of the endometrium or to a delay in the transportation and reception of ova from the tubes to the uterus. It was thought that the injection of a low dose of progesterone may result in delayed implantation. Chang(8) has indicated that the injection of a low dose of progesterone in non-ovulated rabbits for 5 to 6 days, only renders the endometrium similar to that of a rabbit in the 3rd to 4th day of pseudopregnancy. Delayed implantation occurs spontaneously in mice as a result of lactation(15) or may be induced by the administration of ovarian hormones(16). It is possible that implantation was not delayed in the present investigation due to the fact that progesterone is rapidly destroyed (or converted into an inactive form) (17,18), particularly in the ovariectomized rabbit(19). Courrier and Kehl(11) have shown that a definite progesterone:estrogen ratio of 750:1 is required for successful implantation in the ovariectomized rabbit. In the present experiment when the progesterone:estradiol ratio was narrow (250:1 or 20:1), the ova were blocked in the tube and degenerated before reaching the uterus.

It is of great interest to note that the actual number of implantations in the two series of the present experiment did not increase with the increased number of ova in the uterus. This may denote a quantitative basis for the hormonal requirement of implantation. Further experiments are needed to explain the high frequency of embryonic mortality in ovariectomized progesterone-treated rabbits. The role of placental hormones in such animals should not be overlooked.

Summary. The role of ovarian hormones in implantation was studied in rabbits ovariectomized after mating and in ovariectomized pseudopregnant rabbits into which fertilized ova were transplanted. There was no evidence of delayed implantation. One to 3 implantations occurred in ovariectomized rabbits injected with 2 or 25 mg progesterone. The percentage of implantations ranged from 15 to 23 irrespective of dosage or the time at which injection started. Implantation failed to occur when the dose was 0.3 mg progesterone or 0.1 mg estradiol in combination with progesterone. The number of implantations did not increase with the transplantation of ova. This may suggest a quantitative basis for the hormonal requirement at implantation. Hormonal requirements at implantation vary from those needed for subsequent development.

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Evaluation of the Sakaguchi Reaction for Quantitative Determination of Arginine.* (22318)

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Sakaguchi(1) reported a color reaction specific for arginine which several investigators have since attempted to adapt for quantitative use. Weber(2) suggested that under certain conditions the reaction could be modified to give a quantitative colorimetric method. The procedure of Weber was further modified by Jorpes and Thoren(3) and Fisher and Wilhelmi(4), who attempted to reduce the rapid fading of color noted by Weber. MacPherson (5,6) attempted to eliminate color fading by adding urea prior to the development of color and by using a high concentration of chromogenic reagent (α-naphthol) and developing reagent (potassium hypobromite). In our hands, this procedure gave a highly colored blank with no improvement in color stability. Sakaguchi(7) reported that if 8-hydroxyquinoline was used in place of α-naphthol, a more stable color was produced. However, we have been able to detect little difference in the stability of colors formed with either chromogenic agent.

We have evaluated the published methods and modifications of these methods and have derived a procedure for estimating arginine which is highly satisfactory from the points of view of sensitivity and duplicability. Levels of arginine as low as 2 γ can be determined quite accurately.

Experimental. Reagents. Arginine hydrochloride—10 γ arginine/ml; prepared as needed from stock solution containing 605 γ arginine hydrochloride/ml, kept at 5°. This solution is stable for about 1 month. *Sodium hydroxide*—10% solution in water. *8-Hydroxyquinoline*—0.02%; prepared by diluting a 0.2% solution in ethanol with distilled water. *Sodium hypobromite*—1%; prepared by diluting 1 g liquid bromine to 100 ml with 5% sodium hydroxide. This is stable for 1 month in the cold and dark. *Urea*—40% solution in water. *Procedure*—All reagents and solutions should be cooled in ice bath before beginning the determination. To a tube containing 5 ml of the test solution (0—30 γ arginine) are added 1 ml of 0.02% 8-hydroxyquinoline and 1 ml of 10% sodium hydroxide. The solutions are mixed thoroughly and replaced in the ice bath for 2 minutes. Then 0.2 ml of 1% sodium hypobromite is added rapidly to develop the color. After mixing and within 15 seconds, 1 ml of 40% urea is added to destroy excess hypobromite and prevent rapid fading of color. One minute after addition of the hypobromite, 5 ml of cold distilled water are added, the solutions mixed, and the absorption is read at 490-510 mμ. It is important that the sample be read within 5 minutes after development of color. Also the readings should be made at the same time interval after color development in each set of analyses.

Standard Curve. A set of 4 tubes is prepared containing 0, 10, 20, and 30 γ of arginine. The blank is developed first and is used to bring the colorimeter or spectrophotometer to zero. The other tubes are then developed in the order given above and results read and plotted.

Results. A linear relationship exists between optical density of the color developed and concentration of arginine in the range of 0-50 γ of arginine per tube; however, it is recommended that 30 γ be the upper limit when using ordinary colorimeters.

Fig. 1 shows a typical standard curve prepared as described above. This curve is reproducible from one experiment to the next within ±0.5%. In Fig. 2 is presented the

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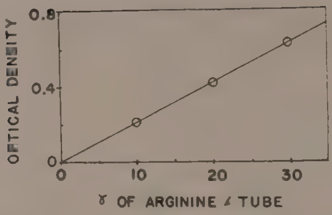

FIG. 1. Typical standard curve for arginine.

absorption spectrum of the color produced in the reaction. It can be seen that the maximum absorption occurs near 506 mμ. Reasonably good quantitative results can be obtained in the range 490-510 mμ, however.

Sakaguchi(1,7) and Poller(8) have studied the nature of compounds which interfere with this color reaction. Glycine appears to give the most interference among the amino acids. Glycine was found to cause a slight color intensification when present in small amounts (up to 100 γ/developing tube) in a solution with arginine. When the concentration of glycine in the developing tube reaches 150 γ or above, however, color fading is noted. In either case if a standard curve is established with a specific amount of glycine, arginine can still be determined.

Our work indicates that arginine may be determined in the presence of mixtures of amino acids. Those we have investigated were amino acid mixtures similar to casein and egg albumin hydrolysates(9). Thus, recovery experiments using amino acid mixtures containing known amounts of arginine indicate that accurate results are easily obtained.

In this laboratory a number of buffers commonly used in biochemical studies (tris(hydroxymethyl)aminomethane, glycine, acetate, phosphate, pyrophosphate, and bicarbonate) were also investigated for possible interference. Only glycine and tris(hydroxymethyl)-aminomethane ("tris") were found to interfere to a measurable extent. It should also be noted that the level of sodium hypobromite is quite critical since an excess tends to cause decomposition of the color produced. Of the series of hypobromite concentration studied. 0.2 ml of 1% sodium hypobromite was found to give full color development and negligible color fading if read within 5 minutes.

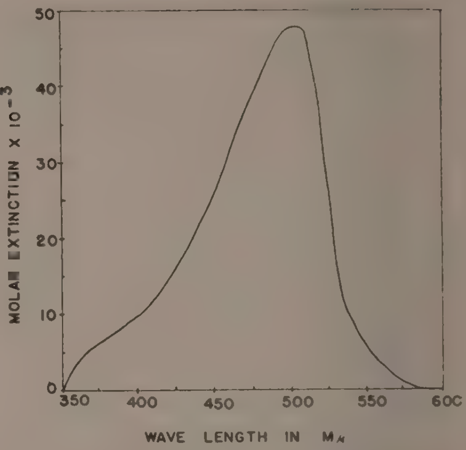

FIG. 2. Absorption spectrum of the colored product formed with arginine.

Summary. The Sakaguchi reaction has been studied to obtain a reliable quantitative method for the colorimetric estimation of arginine. The best conditions for this method have been investigated and reported. The method has been found to be entirely satisfactory for determining arginine over the range of 0-30 γ. Duplicability of the method is ± 0.5%, (Standard deviation).

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Colorimetric Method for Rapid Determination of Arginase Activity.*
(22319)

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Existing methods for determining arginase activity generally involve measuring one of the products of arginine hydrolysis. For example, ornithine has been determined by titration(1). The most usual product measured, however, is urea, which can be estimated by the cumbersome procedure of decomposition with urease followed by the manometric measurement of carbon dioxide(2,3) or the colorimetric(4) and titrimetric(5) estimation of the ammonia produced. Urea can be directly estimated colorimetrically, but arginine has been reported to interfere with the color produced(6). In addition, most of these methods require more arginase activity than many studies allow. We recently had need of a rapid method for arginase determination in which only small amounts of tissues were required. A note by Iyer and Krishna Murti (7) appeared to offer a promising method, in which arginine remaining after hydrolysis was estimated colorimetrically by the Sakaguchi reaction as modified by MacPherson (8). However, the method of Iyer and Krishna Murti(7) could not be reproduced since the details of carrying out the method as well as the results presented could not be adequately interpreted. It was also found that the suggested glycine buffer gave marked inhibition of color development.

In the present paper, we wish to present a method which allows the accurate determination of arginase activity in samples of liver as small as 20 γ (fresh weight).

Methods. Reagents. In addition to those reagents listed by Gilboe and Williams for determining free arginine(9), the following reagents are required for estimating arginase activity: 0.4% trichloroacetic acid (TCA); 0.1 M sodium pyrophosphate adjusted to pH 9.5; and a solution containing 50 γ of arginine per ml, prepared as needed from a stock solution containing 605 γ arginine hydrochloride per ml kept at 5°. The last solution is stable for about one month. *Procedure.* A liver homogenate containing 0.1 mg of liver per ml is prepared by homogenizing a weighed portion of rat liver, obtained from, a freshly sacrificed rat, in ice-cold distilled water. The homogenate should be kept in an ice bath and used within 30 minutes after preparation. In a test tube, large enough for mixing of added solutions by swirling, are placed 3 ml of the diluted arginine solution and 1 ml of the pyrophosphate buffer. The mixture is placed in a constant temperature bath maintained at 37°. After a 5-minute temperature equilibration period, 1 ml of the liver homogenate is added; the contents are mixed quickly, and the mixture is incubated for exactly 10 minutes. At the end of the 10-minute period, 1 ml of the incubation mixture is withdrawn and pipetted into a tube containing 4 ml of 0.4% TCA. The protein precipitated from this amount of tissue is so low that centrifugation is not necessary. However, if larger amounts of tissue are used, for example, tissues containing exceptionally low arginase activity, centrifuging would be necessary to obtain a clear extract. The procedure for estimating the unhydrolyzed arginine has been outlined by Gilboe and Williams(9). A blank is run using the same procedure as above except that the arginine is omitted. Since each tube except the blank contains 30 γ of arginine per ml before incubation, the amount of arginine hydrolyzed is determined by subtracting the γ of arginine per ml remaining in the tube from 30 γ. By decreasing all volumes to one-fifth those given above and adding 4 ml of TCA directly into the incubation mixture, only 20 γ of liver are needed.

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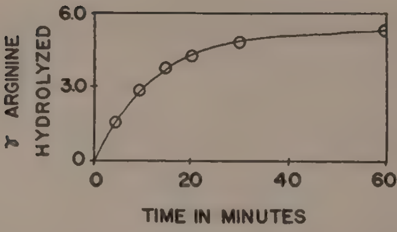

FIG. 1. Rate of arginine hydrolysis in a typical experiment.

A *standard curve* should be established by mixing 0, 1, 2, and 3 ml of the arginine solution (50 γ per ml) in 4 separate tubes, adding 1 ml of 0.1 M sodium pyrophosphate to each tube, diluting to 5 ml with water, and following the procedure outlined above for taking the sample and developing the color. This method of obtaining a standard curve gives both a standard curve and a check on the procedure.

Results. To determine the proper incubation time, a mixture of 6 ml of 50 γ of arginine per ml, 3 ml of the pyrophosphate buffer, 3 ml of distilled water, and 3 ml of the liver homogenate was incubated at 37°. One ml aliquots were taken from the incubation mixture at 5, 10, 15, 30 and 60 minutes. The amount of arginine hydrolyzed was determined as previously described. Fig. 1, which is a plot of the arginine hydrolyzed *vs.* time, indicates there is no longer a straight line relationship between enzyme activity and time after 10 minutes. This observation agrees with the results of Van Slyke and Archibald(6).

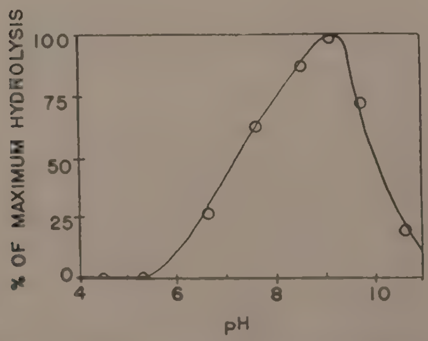

FIG. 2. Effect of pH on arginase activity (avg of 2 experiments).

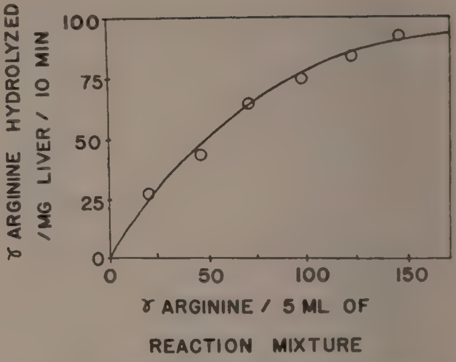

FIG. 3. Arginase activity *vs.* arginine concentration (avg of 7 experiments).

The optimum pH of the enzyme was determined by carrying out the reaction in a compound buffer (0.05 M in each anion) prepared from the following substances: sodium acetate, acetic acid, sodium dihydrogen phosphate, disodium phosphate, sodium pyrophosphate, sodium carbonate, and sodium bicarbonate. Portions of this compound buffer were adjusted to pH 4, 5, 6, 7, 8, 9, 10 and 11 respectively. From Fig. 2 it can be seen that the pH optimum lies between 9.0 and 9.5, which is in agreement with the data of previous workers for arginase(6,10).

The substrate concentration curve for 0.1 mg rat liver per 5 ml total reaction volume is shown in Fig. 3. It can be seen that the curve levels off near 150 γ of arginine. The linearity of the enzyme activity *vs.* enzyme concentration over a 5-fold range using 150 γ arginine is shown in Fig. 4. Thus it may be concluded that the enzyme in 0.1-0.5 mg of liver per 5 ml total reaction volume is saturated when the amount of substrate is 150 γ.

Further experiments have indicated that additional manganese is not needed in the system reported in this paper. This indicates that sufficient manganese is present in the whole liver homogenate to activate the enzyme maximally. Using purified arginase preparations, manganese would undoubtedly need to be added. This is left to the discretion of the operator when preparations other than whole liver homogenates are used. From data in Fig. 3 the Michaelis constant for this

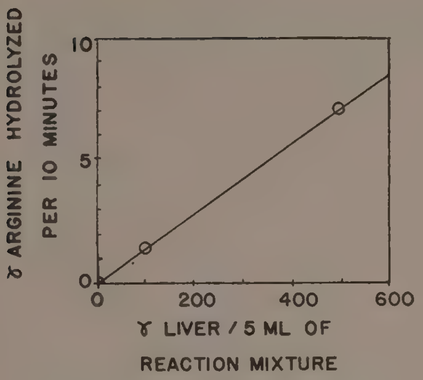

FIG. 4. Linear response of arginase activity *vs.* liver concentration (avg of 2 experiments).

system was calculated to be 1.53 x 10^{-4} M which agrees with a calculation of the Michaelis constant from data presented by Van Slyke and Archibald(6).

Summary. A method has been presented for the rapid determination of arginase with a minimum of operations and equipment. The activity of as low as 20 γ of whole liver can be determined. The arginine remaining after enzymatic hydrolysis is estimated by a quantitative modification(9) of the Sakaguchi reaction. Additional studies are presented indicating the necessary substrate concentration, the pH optimum, and linearity of the enzyme concentration curve for these experimental conditions.

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Effect of Cholinergic Drugs on Development of Chick Embryo.* (22320)

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There is sufficient evidence to indicate the presence of cholinesterase in embryos. Zacks (10) demonstrated the presence of cholinesterase in the embryonic chick from 0-96 hour stages. Wenger(8) followed the development of cholinesterase in spinal cords of chick embryos of 5½ to 11 days of incubation. Nachmansohn(4,5) determined cholinesterase activity in chick nerve and muscle at 6 or 9 days of age. He followed the increasing levels of activity of this enzyme to about the 15th day and showed a higher rate of increase until hatching. Similar observations have been made on early and later stages of amphibian embryos (Youngstrom(9), Sawyer (6,7), Boell and Shen(1,2)).

Since cholinesterase is present during early embryonic stages and increases steadily as the neuromuscular apparatus appears, the administration of enzyme inhibitors and the accumulation of acetylcholine could be attended by developmental abnormalities. To investigate this possibility, 4-day chick embryos were treated with various doses of cholinesterase inhibitors (prostigmine bromide; physostigmine salicylate), the enzyme

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TABLE I. Effects of Acetylcholine (Ac) and Atropine (A) on Development of Chick Embryos.

Drug	Route	Eggs	mg of drug or 0.85 cc NaCl at days of incubation								Mortality				Sac.	Wt (g)	Comments
			4	5	6	7	8	9	11	12	5	6	7	8	13		
Ac	A.s.	2	.5	.25								2					
		2	.5	.25	.5		.4	.4	.4	.4					2	8.5, 7	Viscera ectopic
		1	1.0	.50	.2		.2	.2	.2	.2					1	9	Normal
	Y.s.	2	.3	.3								2					
		2	.25	.25	.3		.25	.25	.25	.25					2	8.6, 9.3	Viscera ectopic
		1	.5	.5							1						
	E.m.	2	.2	.2		.2					1			1			
		1	.2	.2		.2	.2		.2	.2					1	9	Normal
A	Y.s.	1	.1	.1	.1		.1		.1	.1					1	8.9	
		4	.4	.4	.4							2	2				
	E.m.	2	.1	.1							1	1					
		3	.2								3						
		1	.2	.1	.2		.2	.2	.2	.2					1	8	Normal
		3	.5	.5	.1		.1	.1	.1	.1					3	7.5, 8.8, 8.9	”
S	E.m.	2	.2	.2	.2		.2	.2	.2	.2					2	9.8, 10	”
Cn		2													2	12, 12.5	”
Totals		31									6	7	2	1	15		

S, 0.85 saline controls; Cn, untreated embryos; Sac., time of sacrifice in days of incubation; A.s., air sac; Y.s., yolk sac; E.m., on the embryo.

substrate (acetylcholine) and a cholinergic blocking agent (atropine).

Method. Eggs containing vigorous 4-day embryos were obtained from a local hatchery. Various amounts of atropine, acetylcholine, eserine salicylate, and prostigmine bromide, were administered in either the yolk sac, air sac, or directly on the embryo. Since boric acid is used as a preservative in the prostigmine bromide preparation, control embryos were treated with 0.01% boric acid and 0.85% sodium chloride. Animals were sacrificed at 7-13 days of incubation, fixed in Bouin's or DeCastro's fluid and stained with Cajal silver and hematoxylin technics. Preparations with silver were used to examine the nervous system.

Results. Large amounts of acetylcholine (1.20 mg-2.85 mg) injected during the interval of 4-12 days of incubation were well tolerated as were similarly administered large doses of atropine (0.60 mg-1.50 mg). Of 27 embryos treated, 11 survived 13 days' incubation, and except for slight decreases in weight and ectopic viscera all were grossly of normal appearance (Table I).

During the interval of 4-8 days' incubation 42 embryos were treated with physostigmine bromide, eserine salicylate and a combination of physostigmine bromide and acetylcholine (Table II). The total amount of eserine or physostigmine given to each embryo was within the range of 1.0 mg-15.0 mg. Of the above 42 embryos, 22 survived 7-13 days' incubation and in all but 3 of the survivors, a remarkable kyphosis syndrome was observed (Fig. 1-5).

The syndrome as observed in 9-13-day embryos is briefly described (Fig. 1-5). The head was approximately the same size as controls, eyelids were retarded, the lower beak shortened, and the external auditory meatus connected with a fissure extending inferiorly and anteriorly. The meatus was larger than in controls. The neck and trunk were reduced approximately one half in length and this can be accounted for by 3 sharp compensating S-shaped curvatures in the cervical, thoraco-lumbar, and sacral-coccygeal vertebrae, respectively (Fig. 5). Bodies of the vertebrae were compressed and wedge-shaped at levels of sharp bending. Ankylosis of vertebrae was observed at levels where the vertebrae were compressed. Upper and lower extremities were shortened.

Throughout all levels of the spinal cord of

TABLE II. Injection of Prostigmine Bromide (P) and Eserine Salicylate (E) into Yolk Sac of Embryonic Chick and Effects on Development.

Drug	Eggs	Days of incubation																	Sn	
		Drug (mg) or 0.85% NaCl (cc)					Mortality						Sacrificed							
		4	5	6	7	8	5	6	7	8	9	10	7	8	9	10	11	13	+	—
P	8	5.0						1	1				1	2	2	1			5	1
	6	5.0	5.0			5.0						1		1	2	1	1		5	
	6	.5	.5			.5			2					1	1	2			4	
P + Ac	6 P	.5			.5				1		2				1	2			3	
	Ac	.4			.4															
E	8	.5		.5		.5	2			2	1							3	1	2
	8	1.0					1	5	1							1			1	
B	8	.1	.5			.5		4					2		2					4
S	8	.1	.5			.5				2					6					6
Totals	58						3	10	5	4	3	1	3	4	14	7	1	3	19	13

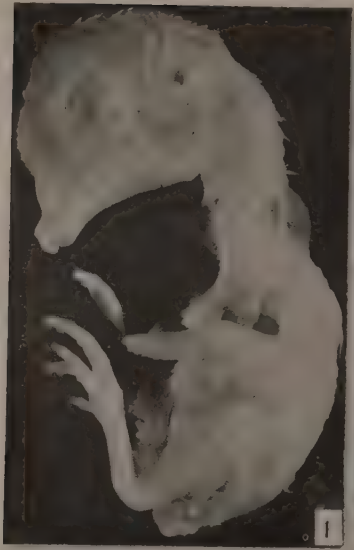
S, 0.85% saline controls; B, 0.10% boric acid; Ac, acetylcholine; Sn, syndrome produced with prostigmine bromide or eserine salicylate (Fig. 4 and 5).

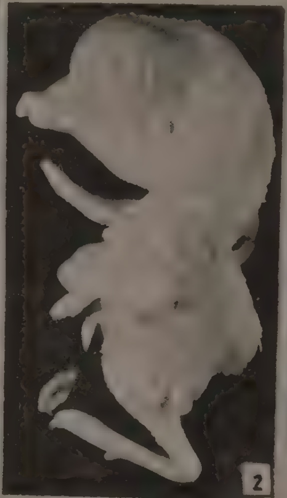
kyphotic animals, the extensive hypoplasia and atrophy of anterior horn cells was most striking. A reduction in volume of musculature was observed along the vertebral column and in the lower extremity. Spinal and sympathetic ganglia were well differentiated but often fused into continuous masses in regions of severe bending. Pedicles and laminae of sacral and coccygeal vertebrae were usually missing. This was generally associated with absence of the spinal cord. Although the brain and brain stem were reduced in size, no gross abnormalities were observed.

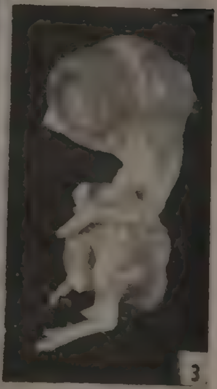
Above experiments were supplemented with observations on 12 embryos injected on the fourth day with .05 mg of physostigmine salicylate. Nine survived 8-12 days' incubation. Of these, 2 were normal, 4 with slight kyphosis at thoracic and lumbar levels; one with normal vertebral column but with shortened lower beak, retarded eyelids and micromelia (Fig. 6); one with no coccyx and with shortened extremities. With high dosages it was typical to find abnormalities in combination with kyphosis. When weaker dosages (.05 mg) were given, the abnormalities were scattered, appearing singly or in unpredictable combinations.

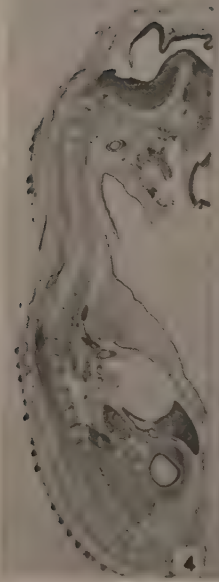
Discussion. Landauer(3) reported the morphological consequences of treatment with sulfanilamide, eserine, and insulin. With heavy doses of these drugs, shortened beaks, micromelia, syndactylism, clubbed down and microphthalmia were observed. Weaker doses produced abnormalities of less degree and a scattering of abnormalities, observed singly or in various combinations. His results were partly confirmed with our observations on embryos treated with weak doses of prostigmine bromide or physostigmine salicylate (Fig. 6). Since similar abnormalities were reported in embryos treated with sulfanilamide and insulin, the mechanism indicated is probably of a non-specific nature.

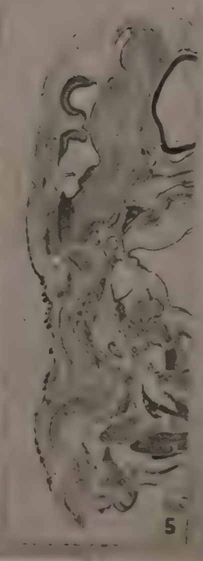
Observations were made by Sawyer(6) on amblystoma embryos reared in various concentrations of eserine (0.01%-0.001%). At Harrison stage 46 a majority of eserinized animals developed one or 2 sharp curvatures of the trunk. There was little retardation of growth, digits were normal, length of gills and height of dorsal fin were decreased. The width of the head was increased. Curvatures of the trunk did not disappear after decapitation or destruction of the spinal cord. This was interpreted as evidence of the muscular origin of the bend reaction. Removal of animals from the inhibitor solution and transfer to water did not eliminate the bend reaction. This persisted as though the skeleton had become fixed in position. Chick embryos eserinized at 4-8 days of incubation subsequently developed extensive kyphosis observed at 7 to 13 days' total incubation. Although this condition seemed more severe than that described for amblystoma by Sawyer(6), the mechan-











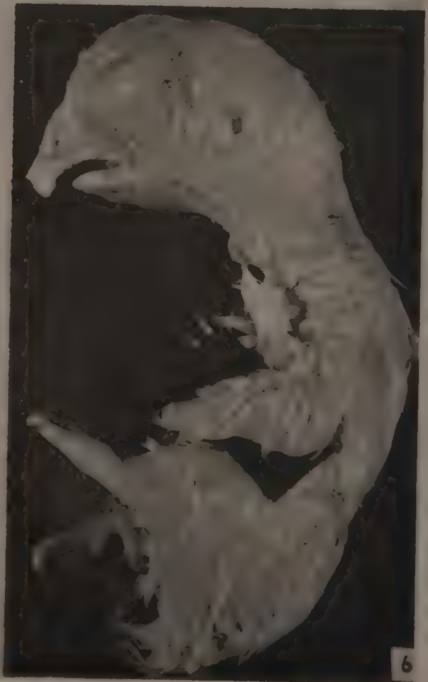


FIG. 1. Control embryo (13-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 0.1, 0.5 and 0.5 cc of 0.85% saline. ×2.

FIG. 2. Embryo (P6, 13-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 5 mg of prostigmine bromide; total dosage 15 mg. ×2.

FIG. 3. Embryo (P1, 8-d) treated (yolk sac) on 4th day of incubation with 5 mg of prostigmine bromide. ×2.

FIG. 4. Mid-sagittal section of control embryo (12-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 0.1, 0.5 and 0.5 cc of 0.85% saline. ×3.4.

FIG. 5. Mid-sagittal section of embryo (12-d) with extensive kyphosis showing 3 compensating S-shaped curvatures. Treated (yolk sac) on 4th, 5th and 8th days of incubation with 5 mg of prostigmine bromide; total dosage 15 mg. ×3.4.

FIG. 6. Embryo with micromelia and shortened lower beak (E-10, 13½-d). Treated (yolk sac) on 4th day of incubation with 0.05 mg of physostigmine salicylate. ×2.

ism of formation may be similar.

It is interesting that the treatment of embryos with large doses of acetylcholine during 4 to 12 days of incubation did not result in malformations similar to those obtained with cholinesterase inhibition. This would suggest the destruction of acetylcholine shortly after its administration. Since cholinesterase increases rapidly as the neuromuscular system develops this is entirely possible. Atropine, a cholinergic block, had no deleterious effect on development.

Summary. 1. Large doses of acetylcholine (1.20-2.85 mg) were injected in the yolk sac, air chamber, or directly on the embryo during the 4-12-day incubation period. These doses were well tolerated by the embryo and attended with no gross abnormalities. Atropine similarly administered produced no malformation. 2. The yolk sac of 4-day embryos was injected with weak doses (0.05 mg) of physostigmine salicylate. Shortened beaks, micromelia, and retarded development of eyelids were observed at 8 to 13 days' incubation. Inasmuch as similar anomalies were reported in sulfanilamide and insulin treated animals, these anomalies cannot be considered eserine specific. 3. With larger doses (1-15 mg) of prostigmine bromide or physostigmine salicylate, the above mentioned anomalies were generally associated with severe midline bendings of the vertebral column (Figs. 4,5), and this was interpreted as specific for cholinesterase inhibition.

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Extraction and Concentration of Thromboplastic Material from Human Urine. (22321)

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Although human urine is known to have thromboplastic property(1,2), little attention has been given to the concentration or purification of the urinary component responsible for this property. There is described herein a simple method for extraction and concentration from normal urine of material with thromboplastic activity, and some characteristics of this material are discussed.

Material and methods. Pooled urine obtained without preservative from normal men is used. The urine, refrigerated not longer than 2 days, is filtered through nonabsorbent cotton, and the pH brought to 4.5 with 2 N HCl. Five g of $BaSO_4$ (x-ray quality) is added to each liter of urine, and the mixture is stirred for 15 min. without being allowed to foam. It is then filtered through large Berkefeld candles type N and the filtrate discarded. Thromboplastic material and a fibrinolysokinase are absorbed on the $BaSO_4$. The $BaSO_4$ is mixed with distilled water (one volume for every 20 volumes of urine), and stirred for 15 min. at a pH of 5.2. The mixture is next centrifuged (20 min. at 1060 *g*). This elution is repeated. The eluates are combined and the sediment set aside for elution of fibrinolysokinase with 3.8% Na-citrate, if desired. The clear, almost colorless eluates are then dialyzed in the cold for two days against distilled water; a slight turbidity forms. All further procedures are carried out at 5°C. The pH is adjusted by a glass electrode to 3.5, and a precipitate is formed. This is collected by centrifugation after standing for one hour and is then dissolved in 1/1,000 of original volume of urine with 1/100 M glycine buffer of pH 9, stirred for 10 min., and stored for one hour. Then the preparation is centrifuged for 30 min. at 2000 *g*. The supernatant is dialyzed by a continuous rocking device, in a cellophane (8/32 inch, Nojax Casing Visking) tubing against running CO_2-free distilled water until pH of 7 is reached. When the supernatant is lyophilized, a white powder soluble to the extent of 1% in 1/100 M glycine buffer of pH 9 is obtained. In solution, it forms a colorless, viscous, slightly opalescent fluid which can be heated to the boiling point (96.5° in Denver) (Table I) with relatively slight loss of thromboplastic activity. This substance is subsequently referred to as the "T" (thromboplastic) fraction. Yield is 2-3 mg/liter.

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* This work was done in part during tenure of Research Fellowship of Amer. Heart Assn. Supported by grants-in-aid from American Heart Assn. and the Belle Bonfils Memorial Blood Bank, Denver.

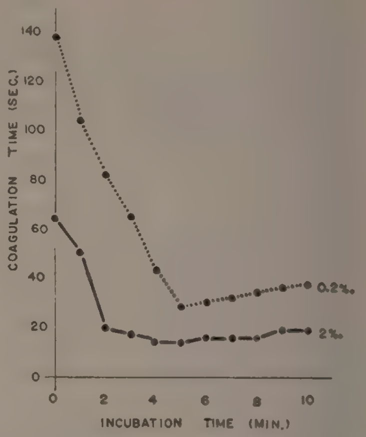

FIG. 1. Thromboplastin generation test. Platelet suspension is replaced by "T" fraction in 1/100 M glycine buffer, pH 9.

Properties. Action of the "T" fraction on hemophilic plasma under various conditions is summarized in Table I. A concentration of only a few micrograms of the "T" fraction per cc of hemophilic plasma is required to restore the clotting process to normal. Heating affects activity of the "T" fraction only to a minor degree, whereas prolonged high-speed centrifugation removes the active material.

The "T" fraction is similar in behavior to

TABLE I. Effect of "T" Fraction on Clotting of Hemophilic Plasma under Various Experimental Conditions. 6.6 μg "T" fraction added/cc plasma.*

Treatment	Recalcification time after addition of "T" fraction (min.)	Prothrombin serum time after addition of "T" fraction (sec.)
2% in glycine buffer, pH 9	9	34.5
Idem; centrifuged 90 min. at 31,000 *g*	46	20
2% in glycine buffer, pH 9	9.3	54
Idem; heated in sealed ampules at 96.5°C, 20 min.	9.5	45

* One μg corresponds to 0.3 ml urine.

Plasma from 2 different subjects has been used to obtain the data for this table. Control recalcification time and prothrombin serum time were 157 min. and 19 sec., respectively, for the first 2 experiments and 58 min. and 20.2 sec. for the last two.

brain extract since both substances contain an incompletely activated thromboplastin. The thromboplastin-like activity of the "T" fraction can be considerably enhanced by incubating it with serum and prothrombin-free plasma, as is done in the thromboplastin generation test(3). Fig. 1 shows that the "T" fraction becomes fully activated after incubation for 4 min. with serum, together with plasma treated with $BaSO_4$.

Discussion. One difference between the "T" fraction and brain thromboplastin is that the latter, when heated, has no effect on the prothrombin consumption of hemophilic blood (4). Another difference is that the "T" fraction can be centrifuged at 2000 *g* for 30 min. without an appreciable reduction in activity. A centrifugal force of 31,000 *g* for 90 min.—conditions similar to those used by Chargaff (5) to purify tissue thromboplastin—is required in order to precipitate this material. Biggs, Douglas and Macfarlane(6) have previously shown that brain extract can be activated by incubation with serum and prothrombin-free plasma—a phenomenon also demonstrable with the "T" fraction. Further studies are indicated in order to determine whether tissue thromboplastin is identical with urine thromboplastin.

Neither the physiologic significance nor the source of the urinary thromboplastic material is known. Using the present method for purification, it is possible to obtain thromboplastin from the very same individual on whom studies are to be performed. Thus, it is no longer necessary to resort to heterologous materials in studies of clotting.

Summary. A method is described whereby thromboplastic material is extracted from normal male human urine by adsorption on $BaSO_4$ and subsequent elution. The thromboplastic material is comparatively resistant to heat; its activity is enhanced by incubation with serum and prothrombin-free plasma. As little as 6 μg/cc of the thromboplastic material per cc of hemophilic plasma was found to restore the clotting properties of this plasma to normal.

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Metabolic Interrelationship between Ascorbic Acid and Pteroylglutamic Acid. (22322)

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A close interrelationship between metabolism of ascorbic acid and pteroylglutamic acid (PGA) was reported by various workers (1-4). Schwartz and Williams(5-7) observed that in PGA deficiency produced by feeding aminopterin, rats excreted less ascorbic acid in urine, and their liver ascorbic acid contents were diminished. This indicated that biosynthesis of ascorbic acid was dependent on PGA-nutrition of animals. Chloretone enhances the biosynthesis of ascorbic acid in rats(8). It was, therefore, of interest to find out if the increased synthesis of ascorbic acid, after chloretone treatment, was interfered with in rats treated with aminopterin. Ascorbic acid helps in the conversion of PGA to citrovorum factor (CF) in rats(9). It was also of interest to find out if this conversion was further stimulated in chloretonised rats.

Methods. Biosynthesis of ascorbic acid. Male rats weighing 100-170 g were fed mineralized whole milk with 2 drops of concen-

TABLE I. 24-Hour Urinary Excretion of Ascorbic Acid by Rats in mg. (Avg excretion of 12 rats.)

	1 day	3-4 day	6-7 day	8-10 day
Milk diet alone	2.5	2.4	2.5	—
+ a*	1.4	1.5	1.4	.7
+ c	1.9	10.4	11.1	11.0
+ c + a	1.9	10.6	12.0	10.4
+ s + c	1.9	9.7	13.2	12.0

* a = aminopterin; c = chloretone; s = sulfaguanidine.

trate of vitamin A and D (Adexolin-Glaxo), twice a week. The animals were divided into 5 groups of 12 animals each. Group I received milk without supplement. Group II were fed 40 γ aminopterin per day. Group III were fed daily 20 mg chloretone dissolved in .2 cc groundnut oil. Group IV were fed both aminopterin and chloretone in doses mentioned above, and Group V were fed daily chloretone and .5 g sulfaguanidine. Within 7 days rats fed aminopterin showed deficiency syndromes. Each animal was placed in a metabolism cage over a glass funnel and urine collected in conical flasks containing 2 cc of a 10% solution of oxalic acid and 1 cc toluene. Total ascorbic acid in 24 hour output of urine was determined by titration against a standardized solution of 2,6-dichlorophenol indophenol(10). Animals of the different groups were killed on tenth day of experiment and ascorbic acid present in liver, kidneys, adrenals, small intestine, testis and pancreas was extracted with 4% sulfosalicylic acid and determined by titration with 2,6-dichlorophenol indophenol(11). Dehydroascorbic acid was absent from these tissues. Thiamine, riboflavin and nicotinic acid contents of liver of all animals were determined(12-14). No significant change in these values in livers from different groups of animals could be observed. The results are given in Tables I and II. *Excretion of citrovorum factor and ascorbic acid.* Ten male rats were divided into 5 pairs, each placed in one metabolism cage and urines collected as described above except when analysed for CF. For CF estimation urines were collected under toluene only. Rats were fed mineralized milk supplemented with 100 γ PGA by stomach tube per animal daily for 2 weeks. Ascorbic acid and CF were estimated in urine samples on alternate days. CF was estimated by the method of Sauberlich and Baumann(15) using leucovorin (Lederle) as the reference standard. Results are given in Table III. Male rats of another group of 12 were similarly fed 100 γ PGA for one week and urinary excretion of CF and ascorbic acid determined. After one week these animals were fed 20 mg chloretone in addition to PGA and urinary excretion of CF and ascorbic acid were determined. The results are given in Table III. *Estimation of CF, ascorbic acid and glutathione contents of livers.* At the end of 2 weeks, rats from each group, described in previous section, were killed by decapitation, livers removed, chilled, weighed, ascorbic acid and glutathione estimated according to the method described previously (11) in aliquots of liver samples and CF according to the method of Doctor *et al.*(16) after incubating aliquots of liver homogenate in nitrogen. The results are given in Table IV.

Results. Administration of aminopterin diminished urinary excretion of ascorbic acid indicating interference in normal synthesis of ascorbic acid in PGA deficiency. Urinary excretion of ascorbic acid was stimulated by administration of chloretone either alone or in conjunction with aminopterin. PGA deficiency, therefore, did not hamper urinary excretion of ascorbic acid in chloretonized

TABLE II. Ascorbic Acid Values of Tissues of Rats (mg/100 g Fresh Tissue).

Rats on	Milk	Milk + a	Milk + c	Milk + c + a	Milk + c + s
Liver	31.2 ± .9*	23.8 ± .4	43.1 ± 2.3	35.8 ± 3.2	42.1 ± 2.2
Kidney	20.9 ± 1.1	15.3 ± 1.4	34.8 ± 1.6	23.5 ± 3.2	35.0 ± 1.7
Adrenals	413.7 ±17.5	274.0 ±14.2	408 ±34.4	294 ±44.5	392 ±46.4
Intestine	46.2 ± 1.2	35.6 ± 2.0	59.8 ± 2.8	45.9 ± 5.1	60.9 ± .6
Testis	24.4 ± .7	23.6 ± .7	26.4 ± 1.0	25.6 ± .1	27.5 ± .4
Pancreas	9.6 ± .4	8.0 ± .7	12.9 ± 1.3	10.4 ± .9	10.8 ± [illegible]

* Stand. error.

TABLE III. 24-Hour Urinary Excretion of CF and Ascorbic Acid by Rats Fed 100 γ PGA per Day.

	Before chloretone		After chloretone	
	CF excretion/animal/day	Ascorbic acid excretion/animal/day	CF excretion/animal/day	Ascorbic acid excretion/animal/day
1st wk	468 ± 44 mγ	.62 ± .06 mg	—	—
2nd wk	566 ± 26	.65 ± .06	1572 ± 163 mγ	6.75 ± .63 mg

TABLE IV. CF, Ascorbic Acid and Glutathione Values of Rat Liver.

	Without chloretone	With chloretone	t
Ascorbic acid	24.3 ± 1.2 mg/100 g	34.6 ± 1.4 mg/100 g	4.7
Glutathione	233.7 ± 8.8 "	273 ± 13.9 "	7.5
CF incubated in nitrogen	151 γ/100 g	143 γ/100 g	

rats. Sulfaguanidine fed rats receiving chloretone also excreted in the urine increased amounts of ascorbic acid. The intestinal flora, therefore, may not be concerned in increased urinary excretion of ascorbic acid.

Ascorbic acid contents of liver, kidney, adrenals and small intestine significantly diminished in animals which received aminopterin. Ascorbic acid contents of these tissues increased when animals were fed chloretone. Administration of aminopterin in addition to chloretone increased ascorbic acid values of these tissues, but the values were at a lower level as compared to values in animals which received only chloretone. These findings indicated that aminopterin-induced PGA deficiency interfered with biosynthesis of ascorbic acid by normal and chloretone-fed rats. There was, however, no change in ascorbic acid contents of testes and pancreas in rats differently treated.

After administration of PGA in normal rats there was 6- to 8-fold increase in urinary excretion of CF. This excretion was further enhanced when animals were fed chloretone. Ascorbic acid and glutathione contents of liver were increased in chloretonized rats but CF contents did not change appreciably. The increased conversion of PGA to CF in chloretone-fed rats might be due to increased synthesis of ascorbic acid and glutathione. Ascorbic acid enhanced urinary excretion of CF in rats fed PGA(9).

Summary. The effect of aminopterin on ascorbic acid excretion and tissue contents of ascorbic acid was studied in both normal and chloretone-fed rats. Aminopterin decreased urinary excretion of ascorbic acid but did not decrease excretion in chloretone-fed rats. Sulfaguanidine also could not diminish the increased ascorbic acid excretion in chloretonized rats. Ascorbic acid contents of liver, kidney, adrenals and small intestine significantly diminished in both groups of animals receiving aminopterin alone or in conjunction with chloretone. Ascorbic acid contents of testes and pancreas of differently treated rats did not change. Chloretone feeding enhanced urinary excretion of ascorbic acid and CF and increased ascorbic acid and glutathione contents of liver.

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In vitro Cultivation of Porcine Kidney. (22323)

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In early tissue culture experiments, choice of a menstruum for proliferation of a virus was generally restricted to host and tissue normally parasitized by the given virus. It is now apparent that these restrictions are not so clearly defined. Enders and his co-workers (1) for instance, propagated poliomyelitis virus in non-neural tissues (kidney, skin-muscle and intestine) and Maitland and Laing (2) grew vaccinia virus on rabbit kidney.

Recent observations that epithelial cells of renal origin support multiplication of a number of viruses beyond the confines of host or tissue specificity prompted us to study the nutritive factors involved in culturing of kidney tissue. Kidney of porcine origin was used because of favorable economic considerations and its availability.

Materials. A. *Porcine Constituents.* 1. *Hog Embryo Extract (HEE).* Embryos (10-20 cm long) were removed from the gravid uteri of sacrificed sows and washed with cold physiological saline. The tails, hooves, heads, ribs, and intestinal tracts were removed and the remaining skin-musculature and organs were cut into small pieces and placed in the deep freeze. The dismembered embryos were thawed 24 hours later and homogenized in a Waring Blendor for 30 seconds. An equal volume of Hanks Balanced Salt Solution containing antibiotics was added and the mixture rehomogenized an additional 15 seconds. The homogenized embryo extract was clarified by cheese cloth filtration and by centrifugation (4,000 rpm for 45 minutes at 1°C). The HEE was sterilized by filtration through an EO (Ertel) pad and stored in the deep freeze in 50 ml amounts. 2. *Hog Serum (HS)* was Seitz filtered and stored in deep freeze in 10 ml amounts until used. 3. *Hog Serum Ultrafiltrate (HSU)* was prepared by forcing hog serum through Visking casing. The pH of HSU was adjusted to 7.3 by means of 0.1 M NaH_2PO_4. The HSU was then Seitz filtered and stored in deep freeze in 10 ml amounts. (Selection of kidney tissue and hog constituents should be made from healthy non-immune stock, otherwise, humoral antibody or endogenous viruses may interfere with virus proliferation.) B. *Bovine Constituents.* Beef embryo extract (BEE), serum (BS), and serum ultrafiltrate (BSU) were obtained from Microbiological Associates, Bethesda, Md. C. *Balanced Salt Solution (BSS)* used in preparation of the media was prepared in the manner specified by Hanks and Wallace(3). Just previous to use, 2.5 ml of a 1.4% solution of sterile $NaHCO_3$ was added/100 ml of BSS. The pH of BSS was approximately 7.4. D. *Synthetic Mixture #199 (SM #199)*, composed of inorganic salts, amino acids, vitamins, nucleic acid derivatives and lipids, was prepared in accordance with procedure recommended by Morgan, Morton and Parker(4). SM #199 was refrigerated until used. E. *Nutritive Media* for initiation of cellular growth consisted of isotonic saline solution (either BSS or SM #199) together with varying amounts of serum, serum ultrafiltrate and embryonic extract. The media were compounded and used within 24 hours. F. *Maintenance Media* were used for cellular maintenance: Hanks-Simms medium was prepared by combining one part of sterile serum ultrafiltrate with 3 parts of BSS. Tenth molar NaH_2PO_4 was used to adjust the pH to 7.5. SM #199 was used without further adjustment. G. *Antibiotics.* Crystalline penicillin G (the potassium salt) and streptomycin (calcium chloride complex) were employed routinely in all media at levels of 250 units and μg/ml respectively. H. *Chick Embryo Extract (CEE)* was prepared in accordance with directions of Syverton *et al.*(5). I. *Hog Serum Fractions* II, IV, V and IV + V were prepared according to methods described by Cohn *et al.*(6) or Cammarata and Deutsch (7).

Methods. An entire kidney was removed from young hog (40-60 lbs. weight) using aseptic technic. The kidney was washed with BSS containing antibiotics. Capsule and macroscopic vasculature were removed and a pie shaped portion consisting of cortical and medullary substance was minced in a Petri dish containing several ml of BSS or SM #199. Tissue fragments were reduced to approximately 1 mm and washed several times with BSS or SM #199 until supernatant was clear. A. *Plasma Clot Technic.* The lower two-thirds of each culture tube (16 x 150 mm) was coated with 0.05 ml chicken plasma (Microbiological Associates). Using a platinum wire inoculating loop, 6-8 fragments of tissue were planted in a single vertical row at 10-15 mm intervals in the plasma coated area. Five hundreths ml of CEE was added and distributed over the tissue embedded in the plasma to induce coagulation. Excess CEE was allowed to drain. Following complete coagulation, 1 ml of nutritive medium was added to each tube. The culture tubes were then capped with white "non-toxic" rubber stoppers (The West Co.) and incubated at 36°C in a roller drum revolving at rate of 12 revolutions/hour. B. *Dry Tube Technic.* Minced tissue suspended in small amount of maintenance medium was introduced into lower half of prewarmed (45°C) test tubes by Wright pipettes. The tubes were inverted to effect drainage and stored at 4°C for 30 minutes. The tubes were then reinverted and 1 ml of nutritive medium was added to each tube. Tubes were stoppered and incubated at 36°C in a roller drum. The dry tube technic was used almost exclusively throughout the investigation when preliminary studies revealed that growth exceeded that of the plasma clot technic while granulation was almost nonexistent. C. *Fluid Change.* When growth was well established (usually within 5 to 7 days) the nutritive medium was removed. Each culture was rinsed twice with 1 ml aliquots of BSS or SM #199. One ml of maintenance medium was added and cultures were reincubated for 5 additional days. The maintenance fluid was changed at intervals of from 4 to 7 days depending upon pH of the medium.

Results. The nutritive media recommended for proliferation of cells in tissue culture are numerous, and vary as to their constituents and percentages in accordance with the organ and species cultured(8,9,10,11,12). To determine the influence of homologous and heterologous constituents of nutritive media on propagation of hog kidney epithelial cells, cultures were grown in media consisting of both beef and hog serum, serum, serum ultrafiltrate and embryo extract. The composition of the various media as well as their influence on growth of hog kidney epithelial cells are shown in Table I. The data derived from this initial study indicated that an all hog nutritive medium was superior to an all beef system and the introduction of any hog constituent into a nutritive medium significantly enhanced rate and extent of growth of hog kidney epithelial cells. Furthermore, growth in a medium composed of hog serum (10%) and SM #199 (90%) excelled that of any

TABLE I. Composition of Nutritive Media Used for Proliferation of Hog Kidney Epithelial Cells in Tissue Culture and Their Influence on Rate and Extent of Growth.

Medium constituent	% composition	Medium No. 1	2	3	4	5	6	7
Serum ultrafiltrate	17.6	B*	H*	H	B	B	—	—
Serum	20.0	B	H	B	H	B	—	—
Embryo extract	10.0	B	H	B	B	H	—	—
Balanced salt sol.	52.4	+	+	+	+	+		
Serum	10.0	—	—	—	—	—	B	H
Synthetic mixture #199	90.0	—	—	—	—	—	+	+
		pH 7.4					pH 7.6	
% of cultures growing†		26	82	75	78	74	56	88
Extent of growth‡		1+	2+	2+	2+	2+	2+	4+

* B—Beef origin; H—Hog origin.

† Within 7 days.

‡ Extent of cellular outgrowth based upon No. of layers of cells radiating from the plant and was evaluated in terms of 1 to 4+.

other media tested.

In accordance with these findings, a more definitive experiment was designed to determine optimum combinations and concentrations of hog constituents to be used in conjunction with SM #199 for growth of kidney cells. The results of this study, summarized in Table II, confirm and stress the growth stimulating role of hog serum in the cultivation of epithelial cells. Cultures grown in a medium containing hog serum were consistently more proliferative than cultures lacking that constituent. HEE and HSU, on the other hand, either alone or in combination with HS did little to influence growth. The data also indicate that a minimum of 7.5% hog serum in SM #199 was necessary to insure good growth.

It is interesting to note that none of the media itemized in Tables I or II adequately supported growth of either porcine splenic or testicular fibroblasts.

Having ascertained that porcine serum was a necessary medium constituent, attempts were made to further reduce the complexity of the nutritive medium by substitution of various porcine serum fractions for whole serum. Epithelial cells were consequently cultured in SM #199 containing serum fractions (II, IV, V, or IV + V) in concentrations equivalent to those found in whole serum (8,9). Findings (Table III) indicate that Fraction II did not initiate or support epithelial growth, and that Fractions IV and V, when used independently, produced growth inferior to that of whole serum. However, when Fraction IV + V was used, growth superior to that of whole serum resulted, suggesting a complementary action. The results also indicate the presence of an inhibitor in serum which is inactivated or removed during fractionation procedures.

It is interesting to note that bovine plasma Fractions IV and/or V have been found to enhance growth of monkey kidney cells in tissue culture(13).

Summary and Conclusions. 1. Some of the nutritional requirements for the *in vitro* culti-

TABLE II. Influence of Different Concentrations of Hog Constituents on Growth of Hog Kidney Epithelial Cells in Tissue Culture.

Medium constituent*	Medium No. 1	2	3	4	5	6	7	8	9	10	11	12
Serum ultrafiltrate	10	5	0	0	0	0	0	0	5	5	0	0
Serum	0	0	10	7.5	5	2.5	0	0	5	0	5	0
Embryo extract	0	0	0	0	0	0	10	5	0	5	5	0
Synthetic mixture #199	90	95	90	92.5	95	97.5	90	95	90	90	90	100
% of cultures growing†	50	35	90	100	70	75	0	0	75	15	78	30
Extent of growth	0	0	4+	4+	3+	3+	0	0	3+	0	2+	0

* In %. † Within 7 days.

TABLE III. Influence of Hog Serum Fractions II, IV, V and IV + V on Growth of Hog Kidney Epithelial Cells in Tissue Culture.

Hog serum fractions*	% of cultures exhibiting various degrees of growth				
	0	1+	2+	3+	4+
II	100	0	0	0	0
IV	11	44	45	0	0
V	43	28	29	0	0
IV + V	0	0	0	0	100
Whole serum	12	13	37	38	0

* In synthetic mixture #199.

vation of hog kidney epithelial cells were determined. The data indicate that cultures grown in a "simple" medium containing Synthetic Mixture #199 (92.5%) and hog serum (7.5%) were consistently more proliferative than cultures cultivated in more complex media containing embryo extract, serum ultrafiltrate and serum of homologous and heterologous origin. 2. None of the media tested adequately supported growth of either splenic or testicular fibroblasts. 3. The influence of various Porcine Serum Fractions (II, IV, V and IV + V) on growth of hog kidney epithelial cells was also investigated. When Fraction IV + V was used, excellent growth was achieved. When Fraction II, IV or V was used independently, growth inferior to that of whole serum resulted.

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Some Metabolic Effects of $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (Meticorten) Administered Orally to Dogs. (22324)

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The discovery of the glucocorticoid and anti-inflammatory activities of Meticorten ($\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione) in experimental animals(1,2) and of its antirheumatic activity in human subjects(3) prompted studies on the metabolic actions of this steroid. Most experimental animal studies with Meticorten have utilized mice or rats. There are, to our knowledge, no published studies of the effects in dogs of large doses of Meticorten on water and nitrogen balance, or of the effects on blood sugar level or on the glucose tolerance curve before and during prolonged oral treatment. The production of a marked polyuria and polydipsia by daily *subcutaneous* treatment of dogs with 10 mg/kg of cortisone has been reported(4). Dogs given the same dose orally did not exhibit these effects. Also, it has been noted(5) that daily intramuscular injections of cortisone (50-300 mg/day) to dogs produced polydipsia and polyuria but there were no changes in fasting blood sugar levels or on the glucose tolerance curves. More recent studies with dogs(6) have confirmed the observations on the absence of effect of cortisone on blood sugar level and also showed a small but apparently significant elevation of R.Q., a decrease in basal metabolic rate, and an increased diuresis and nitrogen excretion during treatment.

Methods and materials. Four intact male beagles were used in this study. Each animal was conditioned to a separate metabolism cage for the entire period of the experiment and was housed under the same conditions for at least 6 months prior to the study. The feeding regimen, diet composition (high nitrogen), and the methods for evaluating nitrogen-balance status have been described previously(7). Meticorten was administered orally, once a day, in a gelatin capsule. Water intake, urine output and specific gravity were measured each morning, and tests for glycosuria and albuminuria were run on aliquots of each 24-hour urine collection. Fasting blood sugar samples were analyzed by a standard method. Oral glucose tolerance tests were run by administering 3.0 g glucose /kg body weight via stomach tube and analyzing blood samples for glucose at 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 hours. The dosage regimen in this study was one in which the steroid dose was increased at intervals until consistent polyuria and polydipsia were obtained and then a gradual reduction of dose was instituted until these changes had disappeared.

Results. Table I summarizes certain of the data obtained with the 4 dogs but does not indicate the rate at which the various changes occur. Fig. 1 and 2 provide some indication of the responses over a period of time and at increasing or decreasing dosage. These figures, representing data for dog No. 18, are quite similar to results obtained with the 3 other dogs used in this study.

It is evident from Table I that with a large dose of steroid it was possible to produce a marked polydipsia and polyuria in all 4 dogs, an increased urinary nitrogen excretion, a decrease in urine specific gravity, and a complete or almost complete eosinopenia. It is also apparent that these effects continued to manifest themselves even as the dose was reduced from the highest level (250 mg/day) to doses of 50-75 mg (Fig. 1).

TABLE I. Effect of Oral Meticorten Treatment on Blood Sugar, Water and Nitrogen Balance.

	Dog No. 17	18	31	32
Before treatment (control)*				
Wt, kg	11.6	10.3	7.6	8.0
N, g N/kg/day	E¶	−.07	−.04	E
Water intake, cc†	200	540	520	800
Urine, cc†	200	260	325	420
Specific gravity	1035	1040	1032	1030
Blood sugar, mg %	55	59	62	67
During treatment‡				
Wt, kg	10.8	9.5	6.5	7.2
N, g N/kg/day	−.35	−.27	−.38	−.45
Water intake, cc	1400	1500	1400	1500
Urine, cc	1400	1610	1250	1620
Specific gravity	1022	1010	1012	1008
Blood sugar, mg %	61.2	59	65	53
Max Meticorten dose, mg/day	250	250	250	250
% fall in eosinophils§	100	100	100	100
After treatment‖				
Wt, kg	11.2	9.5	7.0	7.4
N, g N/kg/day	+.04	+.02	+.08	E
Water intake, cc	100	500	410	250
Urine, cc	100	380	315	150
Specific gravity	1035	1035	1040	1025
Blood sugar, mg %	59	71	55	59

* Twelve-day control period.

† Maximum 24-hr value.

‡ Avg values at maximum dose. Period at this dose 12 days for dogs 18, 31 and 32, and 21 days for dog 17.

§ Compared to "Before treatment (control)."

‖ Post-treatment period 17 days for dogs 18 and 31, and 3 days for dogs 17 and 32.

¶ E = equil.

At no time during these experiments did any animal exhibit hypo- or hyperglycemia, glycosuria or albuminuria. Glucose tolerance curves obtained before and during treatment at the maximum dose level (250 mg/day) were normal in every instance.

We have also confirmed (unpublished observations) the published observations on the effects of cortisone on water metabolism. In our experience, the minimal doses which produce significant eosinopenia do not necessarily produce negative nitrogen balance or polydipsia and polyuria. Similarly, larger doses which produce eosinopenia and negative nitrogen balance do not cause polydipsia or polyuria when the dose is not high (100-150 mg/day). However, with still larger cortisone doses (200-300 mg/day) all these effects usually can be produced, although we

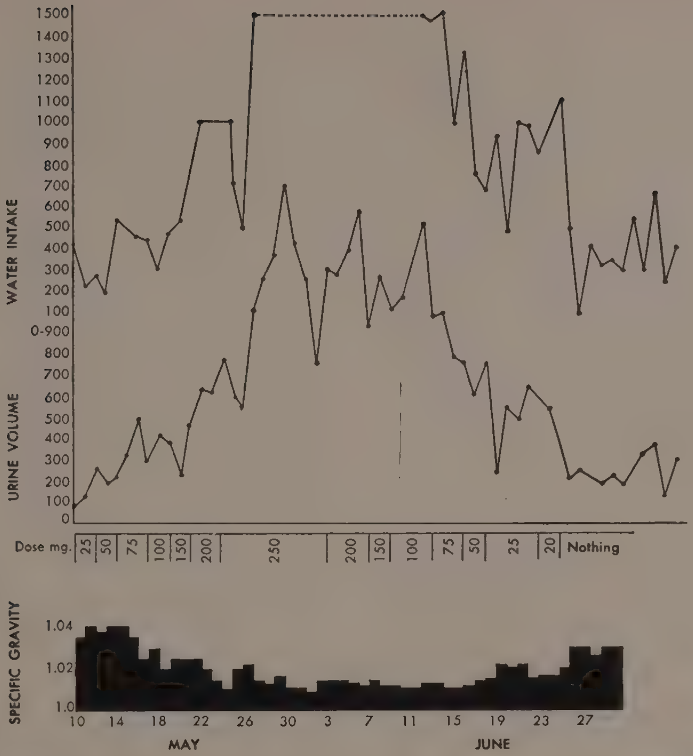

FIG. 1. Effect of Meticorten on water balance and urinary specific gravity in normal dog 18.

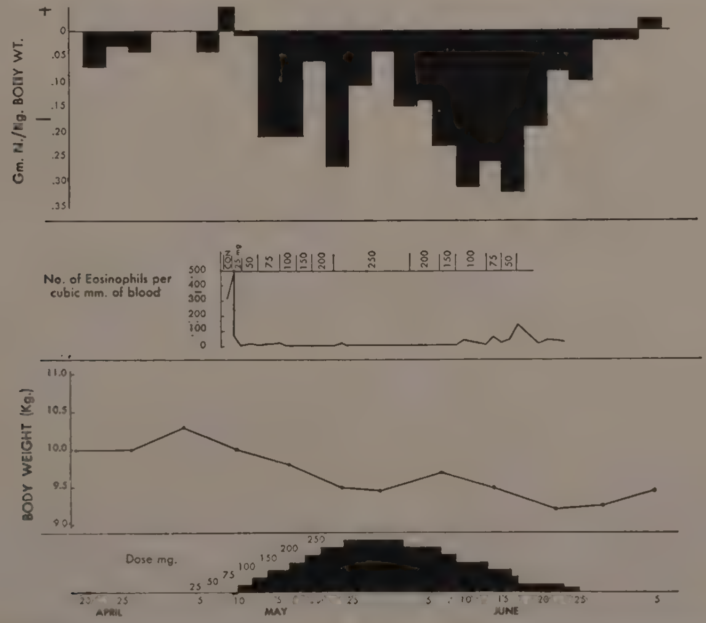

FIG. 2. Effect of Meticorten on nitrogen balance, body wt and eosinophil level in normal dog 18.

have had experience with one male dog which, even at doses producing polydipsia and polyuria as well as negative nitrogen balance, exhibited no change in circulating eosinophil level. Also the sequence in which the various changes (eosinopenia, negative nitrogen balance, polydipsia and polyuria) usually occur would suggest a differential sensitivity of each of these responses to dose.

Summary. 1. In dogs large, prolonged, oral doses of Meticorten induced polydipsia, polyuria, and negative nitrogen balance. 2. Blood glucose levels and glucose tolerance curves were unaffected by the treatment, and no evidence of albuminuria and glycosuria was obtained.

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Protective Action of Glutamine, Cysteine and Other Substances Against Experimental Lathyrism in the Rat. (22325)

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Although the toxic action of sweet peas, *Lathyrus odoratus*, is known to be due to β-aminopropionitrile (occurring as its γ-glutamyl derivative in the sweet pea)(1,2,3), the specific metabolic disturbance caused in the rat by the ingestion of this substance is still undetermined. It is to be expected that discovery of a specific compound having a protective action against sweet pea toxin will aid in revealing the metabolic site at which the toxin acts. The finding of a protective agent may also be of considerable importance because of the possible relationship between odoratism (sweet pea lathyrism) and a number of human diseases of unknown etiology(4,5).

Methods. Male, albino, weanling rats, 24-28 days of age and weighing 48-64 g were used in all experiments. All rats were Sprague-Dawley (Holtzman) strain. Because all young rats receiving substantial amounts of sweet pea meal in their diets rapidly develop lathyrism without exception, screening tests for protective agents can be conducted with a relatively small number of rats. For screening purposes, therefore, supplements were usually fed to 3, but sometimes 2, rats at each level. *Diets*. In a few of the earlier experiments the basal diet contained 50% sweet pea meal* and 25% corn starch and was made up as previously described(6). In most of the screening tests, however, the basal diet contained 30% sweet pea meal and 45% corn starch. Supplements were incorporated by replacing an equivalent amount of starch. All diets contained 10% casein (not vitamin-free) except as noted below. In a few instances supplements were added to Rockland Rat Diet containing 0.1% of synthetic β-aminopropionitrile hydrochloride.† Since high levels of casein exhibit some protective action(8,9,10) the following device was used with supplements which appeared to have anti-lathyrismic properties. Five per cent of supplement was incorporated into a diet containing 30%

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* Sweet peas were generously supplied by Mr. Raymond H. Coulter of Ferry-Morse Seed Co.

† β-aminopropionitrile was synthesized by the method of Buc(7), then converted to hydrochloride and crystallized from 95% alcohol.

sweet peas and 30% casein. Rats receiving this diet were compared to rats receiving unsupplemented diet containing 30% sweet peas and 35% casein. If the supplement had appreciable protective activity, it could be expected to cause improvement above that produced by the increment of casein which it replaced.

Evaluation of Supplements. The animals were weighed weekly or oftener. Observations of muscle tone, activity, etc. were made at frequent intervals. The primary criteria for determining the severity of the advancing lathyrism, however, were evaluations of skeletal changes. These were made by palpation of mandibles, spine, and thorax(8). Levels of supplements which cause decreased growth rate sometimes appear to have protective action due to the fact that, other things being equal, the more slowly growing animal develops skeletal deformities at a slower rate. For this reason it was necessary to differentiate between a more slowly developing lathyrism due to decreased food consumption and a positive protective action. Because assessment of severity of the symptoms was subjective, and because there is some individual variation in the rate of progress of the disease, only those supplements which caused an unquestioned improvement definitely outside the symptom complex bracketed by control animals were considered to exert any protective action. Supplements which appeared to be beneficial, but caused an improvement which could conceivably lie within extremes of individual variation, were fed to more rats at the same level as before and at higher levels. Supplements which were found toxic at the levels fed (severe growth inhibition or death) were re-tested at lower levels.

Results. Partial protection shown by high levels of *protein* (casein and gelatin) have already been reported(8). Casein hydrolysate (enzymatic) fed at a 30% level (with 10% casein) showed some protective activity, although it proved to be less protective than casein itself. Lactalbumin was almost equal to casein in protective activity. Zein exerted no protective action. Zein at 30% (with 10% casein), in spite of its amino acid deficiencies, had no growth-inhibiting effect; its effects on growth and on development of lathyrism could not be distinguished from that of starch.

When 5% *L-cysteine-HCl* was incorporated into the basal diet, some inhibition of growth resulted. A 4% level permitted growth comparable to that obtained with the unsupplemented basal diet. Delayed development especially of mandibular exostoses could readily be distinguished by palpation for the first 2 or 3 weeks after weanling rats were placed on diet. This protective action was temporary, however, for at 3 to 4 weeks cysteine-fed rats were no better off than controls, *i.e.*, cysteine did not decrease eventual severity of the skeletal changes but only delayed their onset. At 2.5% cysteine-HCl gave only very slight, barely perceptible, protection. When L-monosodium glutamate (10%), L-monosodium glutamate plus glycine (10% + 5%), L-leucine (10%), L-lysine-HCl (10%), glucosamine-HCl (5%), or pyridoxine-HCl (0.5%) were added to the basal diet containing 4 or 5% of L-cysteine-HCl, no protection over that due to cysteine was obtained. A diet containing 5% cysteine-HCl plus 30% casein gave somewhat better protection than a diet containing 35% casein without added cysteine. The high level of casein in this diet overcame the growth-depressant effect of cysteine. A level of 5% of cysteine-HCl gave significant protection also to rats receiving 0.1% of β-aminopropionitrile-HCl in Rockland Rat Diet, although the growth-depressant effect of the cysteine was here again in evidence. Addition of 5% *L-glutamine* to basal sweet pea diet also caused delay in onset of skeletal changes. In contrast to the growth-depressant effect of cysteine, glutamine had a growth-stimulating effect. The protective action of glutamine was of longer duration than that of cysteine. Five weeks after beginning of experiment, although skeletal deformities by this time were marked, rats receiving glutamine were not only larger than control rats, but their fur was sleeker, they had better muscle tone and were more active. A 10% level of glutamine gave no more protection than did a 5% level. A diet containing 5% glutamine plus 30% casein seemed to give slightly better protection than a diet containing 35% casein without added glutamine, however the beneficial effect of glutamine in this diet was not clear-cut. Glutamine (5%) added to Rockland Rat Diet containing 0.1% β-aminopropionitrile delayed skeletal changes during the first 2 weeks of the feeding trial. After 3 weeks on this diet, however, there was no detectable difference between these rats and rats receiving the same diet without added glutamine. Rats receiving Rockland diet containing the aminopropionitrile grew better than rats receiving sweet pea diet. Glutamine had no growth-stimulating effect in the Rockland diet.

Addition of 5% L-cysteine-HCl plus 5% L-glutamine to basal sweet pea diet had growth-stimulating effect, but protection against skeletal changes was no greater than with either of these supplements alone. Pyridoxine-HCl seemed to vitiate the beneficial effects of glutamine; rats receiving 5% L-glutamine plus 0.5% pyridoxine did no better than rats receiving the unsupplemented basal ration.

The following *amino acids* were not protective: L-lysine-HCl (10%), L-leucine (10%), L-cystine (5%), L-monosodium glutamate (up to 20%), L-glutamic acid (10%), L-ammonium glutamate (10%), L-histidine-HCl (5%-growth inhibition), DL-aspartic acid (10%—some growth inhibition), L-asparagine (10%), β-alanine (5%), DL-methionine (2%; 10%—toxic), glycine (5%), and DL-Serine (4%; 10%—toxic). The partially protective action of lysine reported by others (11) was not confirmed in this study.

The following *carbohydrate derivatives* were found to have no protective action: glucosamine hydrochloride (5%; 15%—toxic), glucuronic acid lactone (5%), chondroitin sulfate (5%), glucosamine-HCl plus chondroitin sulfate (5% + 5%), and sodium glucuronate (10%). Since there was some question as to whether or not glucosamine-HCl exerted slight protective action at the 5% level, and because cysteine was later found to be somewhat protective, glucosamine-HCl and L-cystine HCl (5% + 5%) were fed together. Glucosamine did not provide any protection above that due to cysteine.

None of the following *vitamins* gave any protection: pyridoxine-HCl (0.5%), Ca pantothenate (various levels up to 5%), L-ascorbic acid (50 mg/100 g), Ca pantothenate plus L-ascorbic acid (20 mg + 50 mg/100 g). Liver powder (1:20, GBI) at 4% level plus added pantothenate and ascorbic acid was ineffective.

The following *miscellaneous supplements* gave no protection: monoethanolamine-HCl (up to 10%—levels of 5% and above caused severe growth inhibition), taurine (5%), sodium acetate (5%), sodium propionate (5%), sodium pyruvate (1.5%), betaine (1%), brewers yeast (15%; 30%—toxic), ribose nucleic acid (10%), sodium glycollate (2%), sodium thioglycollate (up to 1%—growth inhibition), sodium thiocyanate (up to 0.5%), ammonium chloride (4%), prostigmine bromide‡ (up to 3.5 mg/100 g), glutathione (100 mg of monosodium salt/day by subcutaneous injection), and cortisone acetate (0.5 mg/day by subcutaneous injection). An alcoholic extract of casein was prepared by continuously extracting casein with 95% ethyl alcohol for 8 hours. When this extract was added in amounts equivalent to 100 g of casein per 100 g of basal diet (modified to contain 30% casein) no additional protection was obtained.

Discussion. The metabolic site at which sweet pea toxin acts is still obscure. That the metabolism of sulfur is in some way involved seems likely. Protein synthesis itself does not seem to be disturbed, since the animals grow relatively well until the terminal phase of the disease is approached and since no toxic lesions can be demonstrated in liver, kidney or other organs(4,13).

Glutamine also appears to be involved in the metabolic defect. Because glutamate does not seem to be able to substitute for glutamine, it might be inferred that the presence of the amide group of glutamine is critical. However, the interference is not merely one of γ-amidation of glutamate, because methionine sulfoxide, which is known to interfere with γ-amidation of glutamic acid, does not give rise to a syndrome comparable to odoratism even when administered to rats in large doses (rats fed 1% methionine sulfoxide grew normally).[§] A clue to the problem may lie in recent publications of Boström, Rodén, and Vestermark(14,15) who reported that glutamine, but not glutamic acid, appears to act as an accelerator of chondroitin sulfate synthesis and probably plays a part in biosynthesis of mammalian mucopolysaccharides. In this connection, it has been pointed out repeatedly that sweet pea lathyrism in the rat seems specifically to involve mesodermal tissues and that the metabolic defect appears to be locallized in the ground substance, which is rich in mucopolysaccharides (*cf.*, for example, 4 and 16).

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‡ Prostigmine bromide was generously supplied by Dr. M. J. Schiffrin, Hoffmann-LaRoche, Inc. Prostigmine methylsulfate has been reported to be effective in treatment of human lathyrism(12).

Conclusions. Of a wide variety of supplements tested for their protective action against development of skeletal deformities in rats fed sweet peas or β-aminopropionitrile, none gave complete protection. Only L-cysteine-HCl, L-glutamine, and proteins were partially protective. Of the proteins tried, casein, gelatin, lactalbumin and casein hydrolysate gave some protection; zein did not. The protective action of glutamine may be related to its probable role in chondroitin sulfate and mucopolysaccharide synthesis.

Note added in press. Experiments completed since this paper was submitted for publication indicate that the protection afforded by L-cysteine-HCl is prolonged if the diet is made up fresh weekly. Under these conditions the protection against development of skeletal changes was greater with cysteine than with glutamine. The decreased protective activity of L-cysteine in an aging diet is probably due to a gradual loss of cysteine because of the lability of its sulfhydryl group.

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[§] Unpublished data.

Relation of Brain Stem to Renal Electrolyte Excretion.[*] (22326)

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Experimental evidence that a cerebral lesion might affect formation of urine was first presented by Claude Bernard. He produced polyuria without glycosuria in the experimental animal by puncturing the median eminence of the floor of the fourth ventricle slightly cephalad to the point where puncture caused polyuria and glycosuria(1). The author's interest in the relationship between the lower brain stem and renal excretion of electrolytes was aroused by study of a patient with probable "cerebral salt-wasting syndrome," due to tumor within the fourth ventricle of the brain. The syndrome consists of persistent loss of sodium and chloride in the urine, despite low concentration of these ions in the blood. In 4 of 6 cases in medical literature (excluding tuberculosis and tuberculous meningitis), patients had diffuse diseases of the central nervous system(2,3). One patient had glioma of the posterior thalamus, with an area of demyelination extending down the brain stem(4). In the sixth case, a brain tumor was present, involving the floor of the fourth ventricle just rostral to the obex(2).

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These cases raise the question whether the lower brain stem may exert any control over electrolyte excretion. The following experiment was carried out in an attempt to clarify this problem.

Method. Ten adult female mongrel dogs were used. Anesthesia was induced and maintained by intravenous pentobarbital. An indwelling urethral catheter was left in place throughout each experiment. When intravenous fluids were administered, a continuous gravity drip was started immediately after catheterization. Using aseptic technic, the *cerebellar fossa was opened.* The lower portion of the cerebellar vermis and the medulla were exposed. The lower portion of the fourth ventricle was visualized by retraction or resection of the lower vermis (results were the same whether or not the vermis was resected). Stimulation of the caudal portion of the floor of the fourth ventricle just cephalad to the obex was then carried out by the Grass model S4B stimulator and bipolar wire electrodes. Parameters of stimulation generally were 0.5 volts, 5/sec. (monophasic), 1 millisec. duration. During stimulation, the voltage was momentarily increased several times. Generalized clonic movements usually appeared at 0.8 to 1 volt. *Urine collection* periods were generally 20 to 30 minutes during control and stimulation. All urine secreted during stimulation was collected as one specimen. Collection periods during operative procedure were generally 1 hour. Urine sodium and potassium content was determined with Baird flame photometer using lithium as internal standard. Chloride was determined by the method of Sanderson(5). The pH was measured on Beckman pH meter.

Results. Complete data from 3 representative experiments are given in Table I. Three

TABLE I. Effect of Stimulation of Caudal Fourth Ventricle Floor on Sodium, Potassium and Chloride Excretion and Urine Volume in Dogs (3 Representative Experiments).

Dog No.			cc/min.	Na	K	Cl	Plasma Na (mEq/l)
				(μEq/min.)			
17	No intrav. fluids	Operative 9:30-10:30	.10	12	9	10	
		Control 10:30-10:55	.08	10	7	3	
		Stim. 11:17-11:47 .5 v, 5/sec.	.40	90	18	20	(Vermis resected)
		Closure 11:55-12:25	.27	50	11	14	
200	Intrav. 5% NaCl	Operative 1:10-2:10	2.4	529	41	575	
		Stim. 2:40-3:10 .5 v, 5/sec.	4.4	1090	79	1160	
		After coagulation of floor of 4th ventricle 3:17-3:47	2.1	570	44	607	
144	Intrav. 5% dextrose in water	Operative 9:45-10:45	1.25	210	26	220	Preop. 144
		Stim. 11:20-11:45 .5 v, 5/sec.	4.52	537	44	551	Before stim. 137
		After stim. 12:00-12:30	.9	41	17	58	After stim. 134
		Close 12:30-1:00	.7	12	12	15	(Vermis resected)

dogs received no intravenous fluids. In one instance, sodium and chloride excretion increased from 10 μEq/min. and 3 μEq/min. respectively before stimulation to 90 μEq/min. and 20 μEq/min. during stimulation. In the second, the sodium excretion rose from 12 to 85 μEq/min. and chloride from 8 to 101 μEq/min. during stimulation. Potassium excretion increased, but not as much as sodium or chloride.‡ Urine volumes also rose. The effect tended to persist in the immediate post-stimulation period. In the third animal, the increase in sodium excretion was insignificant during stimulation with 0.5 volt, but increasing the parameters to 1 volt, 10 per second, resulted in a small *decrease* in sodium excretion and urine volume. During this latter period, there were continuous clonic movements of the suboccipital muscles.

One animal received a continuous intravenous infusion of 5% sodium chloride. The output of sodium, potassium and chloride doubled during stimulation of the floor of the fourth ventricle, and urine volume almost doubled (Table I). Following stimulation, the superficial caudal portion of the floor of the fourth ventricle was destroyed with electrocautery. During the next 20 minutes, excretion rates were very close to pre-stimulation values.

Continuous intravenous infusions of 5% glucose in distilled water were administered by gravity to 6 dogs. In two animals, stimulation resulted in marked increase in sodium excretion (59 to 234 μEq/min. and 210 to 537 μEq/min.) and chloride excretion (58 to 196 μEq/min. and 220 to 551 μEq/min.). There was a rise in urine volume, and very little change in potassium excretion.§ In a third animal, sodium and chloride excretion increased slightly with stimulation at 1.5 volts, 10/sec.

In the remaining 3 dogs, the results varied. In Dog No. 101, sodium and chloride excretion increased with the electrodes in place, without electrical stimulation. The pressure of the electrodes may have acted as a stimulus. Stimulation at 0.2 volt resulted in a decrease in electrolyte excretion. There was little change at 0.5 volt, but when the stimulus was increased to 1 volt, a definite decrease in sodium and chloride output occurred. In Dogs No. 149 and 151, stimulation was accompanied by a decrease in sodium and chloride excretion, with an increase following stimulation in No. 151.

Two additional experiments were carried out, but are not listed. In 2 dogs, the orbital surface and the convexity of the frontal lobe were stimulated at varying voltages. One dog received 5% dextrose in distilled water, the other 5% sodium chloride. No definite change in urine volume or electrolyte excretion was noted.

Discussion. The bipolar method of stimulation used in these studies probably results in stimulation of a relatively large cross-sectional area of the brain stem at the level stimulated, depending on voltage. Thus at 0.7 to 1 volt, clonic movements of the extremities occurred, presumably from stimulation of the pyramidal tracts. It was noted, however, that voltage threshold for clonic movements varied from one animal to the next, suggesting that at the voltage selected (0.5 volt), the spread of the stimulus varied somewhat among the animals used. This may explain in part the variability of the results.

The data obtained, however, suggest that an influence may be exerted by the brain stem on renal excretion of electrolytes, particularly sodium and chloride. In 5 dogs, stimulation of the caudal portion of the floor of the fourth ventricle at 0.5 volt, 5 cycles per second, resulted in increased excretion of sodium and chloride. A smaller increase in potassium excretion also occurred in these dogs. Slight rises of questionable significance occurred in 3 dogs, and there was some decrease in excretion rate in 2 dogs. Increasing the stimulus strength to 1 volt (and thereby presumably increasing the spread of the stimulus) in 2 dogs resulted in a decrease in excretion rates.

Previous studies of the effects of brain stimulation(6), increased intracranial pressure(7,8) and splanchnicotomy(9-11) suggest that the nervous system may influence renal electrolyte excretion. The author is not aware of studies similar to the present one.

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‡ Concentration of sodium and chloride in urine (mEq./l.) also increased during stimulation, while concentration of potassium decreased.

§ In Dog No. 144, see Table, the serum sodium had fallen from 144 mEq./l. to 137 mEq./l. prior to stimulation.

These results are preliminary. Further studies are planned.

Summary. In anesthetized dogs, bipolar stimulation (0.5 volt, 5/sec.) of the caudal portion of the floor of the fourth ventricle resulted in increased renal excretion of sodium and chloride, and a smaller increase in potassium excretion in 5 instances, slight changes in 3, and decreased excretion in 2. In 2 dogs, increasing the stimulus to 1 volt resulted in a decreased excretion rate of these electrolytes.

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Identification of Cystine Calculi in Mink. (22327)

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Leoschke, Zikria and Elvehjem(1) in quantitative analyses of 31 urinary calculi from mink revealed the main constituent to be magnesium ammonium phosphate, accompanied by small amounts of calcium phosphate and magnesium phosphate. Sompolinsky(2), on the basis of qualitative analyses of 26 stones classified 12 as pure magnesium ammonium phosphate, 13 as magnesium ammonium phosphate plus calcium and 1 as magnesium ammonium phosphate plus calcium and urate.

Observations on bladder stones from mink submitted to the Veterinary Diagnostic Laboratory at Oregon State College have generally indicated an inorganic-type structure. Recently, however, a male mink was presented whose bladder contained 140 small creamy-white colored, smooth, irregularly-shaped calculi and these when submitted to normal ashing procedures proved to be wholly organic matter. Total oven dry weight of the calculi was 1.023 g. The appearance and size of these calculi are indicated in Fig. 1.

Observations. Qualitative analyses of these stones were carried out in the sequence proposed by Hawk, Oser and Summerson(3). These tests indicated strongly the presence of cystine.

Further identification was made by means of Sullivan's reaction which is reported to be specific for cystine(4). A few mg of powdered calculus were taken up in 5 ml of approximately 0.1 N HCl and 1 ml each of 1% NaCN in 0.8 N NaOH and 0.5% aqueous 1, 2-napthoquinone-4-sodium sulfonate were added. These were followed by the addition of 5 ml 15% Na_2SO_3 in 0.5 N NaOH and the mixture was allowed to stand 30 minutes. On

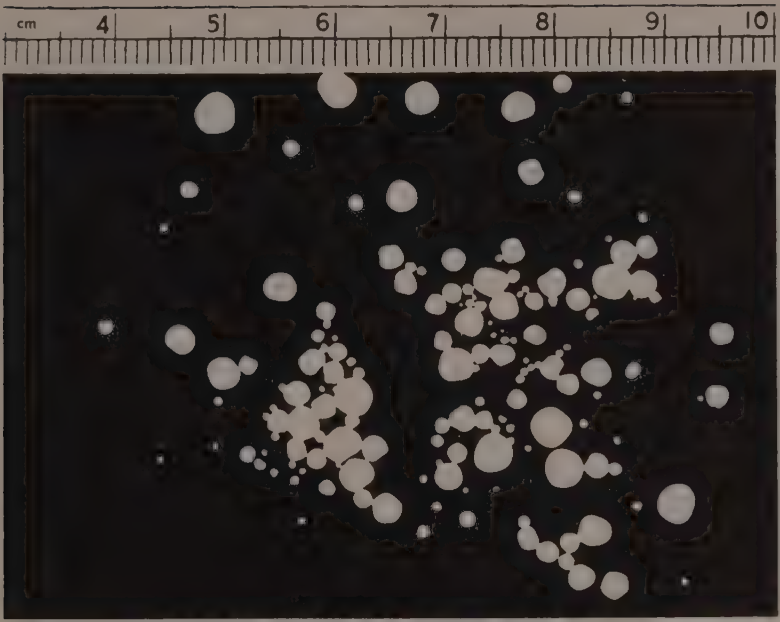

FIG. 1. Cystine calculi from the bladder of a mink.

addition of 1 ml of 2% aqueous sodium dithionite ($Na_2S_2O_4$) a clear red color developed. This test was checked against pure DL cystine with positive results.

Finally, a quantitative test for cystine was carried out upon the dried, ground calculus material, following the method of Lugg(5), using a phosphotungstic acid reagent prepared according to directions of Folin and Marenzi (6). This test revealed the stones to be essentially completely (99%) cystine.

Discussion. Occurrence of cystine calculi in the bladder of dogs, first noted by Lassaigne in 1823(7), has since been reported by several workers(8-11), however, no reference to this type of stone in mink has been found in the literature. It is interesting that the case cited herein involved a male mink, since studies with humans and with dogs(8) have indicated that cystinuria is mainly confined to the male sex, however, this may be due to the constriction of the urethra in the male, where even small stones of the cystine type might cause stoppage.

The diet fed on the ranch where the cystine calculi occurred was not unusual, consisting of approximately 55% Pacific coast fish, 25% tripe, 10% liver and 10% commercially prepared cereal mixture. Since rations fed ranch mink are customarily high in animal protein, however, there may be some dietary predisposition towards high cystine excretion.

Summary. One hundred forty small (0.3 to 3.1 mm dia.) calculi were obtained from the bladder of a male mink on autopsy. Both qualitative and quantitative analyses of these stones indicated that they were almost entirely composed of cystine.

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Effect of Lyophilization and Chloroform Extraction on Electrophoretic Migration of Serum Lipoproteins.* (22328)

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It has been shown that when serum from normal human subjects was lyophilized and extracted with cold chloroform for 3 hours only a small percentage of the total serum cholesterol was extracted(1). This fraction has been designated the "readily extractable cholesterol"—REC. The value for the REC fraction was shown to be 40 mg % or less in 98% of 687 subjects between the ages of 17-33 years, but the percentage of subjects with extraction values above 40 mg % was found to increase up to about the fifth decade(2,3). This was especially marked in the executive and professional groups studied. Subjects with coronary infarction or with atherosclerotic heart disease showed a still higher percentage with elevated extractions.

In an attempt to obtain information concerning the lipoprotein fractions from which the cholesterol was extracted, paper electrophoretic studies were carried out on a) original serum, b) reconstituted lyophilized serum, and c) reconstituted chloroform-extracted, lyophilized serum. Lyophilized sera were reconstituted by the addition of an amount of water equal to the original volume of serum, namely, 0.5 ml. Since the volume occupied by the serum solids was ignored, the concentration of the various substances in these solutions was slightly less than in the original serum.

Method. A Kelab paper electrophoresis apparatus was employed using Whatman #3 filter paper strips (4.6 x 35 cm) with a barbital-sodium acetate buffer of pH 8.6 and ionic strength .05. Three paper strips separated by plastic strips were used in each of the 2 compartments of the apparatus, thus making a total of 6 strips for each run. To each of the strips at a line 12 cm from the cathode end, 0.05 ml of the test solution was applied. The electrophoresis was run for 15 hours at 5°C unless otherwise stated, with 1.2 ma per strip and a potential of approximately 210 volts. The strips were removed from the apparatus at the end of the electrophoretic procedure and processed as follows: 2 strips were dried at 100°C for 45 minutes, then stained for protein with bromphenol blue according to the method of Köiw, *et al.*(4); 1 strip was air dried and stained for lipids with Sudan black-B; and the remaining 3 strips were kept moist by suspending them in a 1000 ml cylinder containing about half an inch of water and covered with an inverted Petri dish to reduce evaporation. As soon as the position of the proteins along the stained strips was determined, which seldom exceeded 90 minutes from the time the strips were suspended in the cylinder, the wet strips were cut as indicated below, the sections transferred to 25 ml glass-stoppered Erlenmeyer flasks, and approximately 17 ml of a 1:1 mixture of ethyl alcohol and ethyl ether added to each flask. Eight sections were prepared: 1) the albumin area which also included the alpha$_1$-globulins, 2) the area between the al-

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bumin + alpha$_1$-globulins and alpha$_2$-globulins, 3) the alpha$_2$-globulins, with the cut being made half way between the alpha$_2$- and beta-globulins, 4) the beta-globulins, 5) a section between the beta-globulins and 5 mm short of the starting point, 6) a section including the area 5 mm on either side of the starting point which usually included some of the gamma-globulins, 7) the remainder of the gamma-globulins, and 8) a 10 mm section towards the end of the strip to serve as a blank. The amount of cholesterol extracted from these sections was determined using a procedure described previously(5).

Results. Effects of lyophilization. The experimental results are shown in Table I. The values for sections 1 and 2 as well as for sections 6 and 7 have been combined. It will be seen that lyophilization invariably increased the amount of cholesterol remaining around the starting point while decreasing that found in the area of the beta-globulins. The effect was most noticeable in those sera with markedly elevated REC values. The REC concentration seems to be of greater significance in this regard than the total cholesterol concentration since, on the whole, the percentage of the total cholesterol which remained around the starting point in the case of the lyophilized sera was greater in Group 3 than in Groups 1 and 2. Each of these groups showed normal total cholesterol concentration but the REC value was quite elevated in the case of Group 3. The percentage of the total cholesterol present in the albumin + alpha$_1$-globulin area was distinctly greater in those subjects with normal REC values than in those with markedly elevated REC concentrations. The rate of movement of the cholesterol associated with albumin and alpha$_1$-globulins was usually not affected by lyophilization. Actually only one of the 21 sera tested showed a definite drop in the cholesterol content of the albumin + alpha$_1$-globulin area following lyophilization. Apparently lyophilization affects primarily the migration of the cholesterol normally present in the general area of the beta-globulins.

Extractability of the lyophilized serum by cold chloroform: Experimental data on the effect of chloroform extraction on the cholesterol distribution are shown in Table II. The values from sections 1-3 have been combined and also those of sections 6 and 7. The sera studied fall into 3 main groups: a) those with normal REC values, b) those with fairly elevated REC values and c) those from which the cold chloroform extracted a large percentage of the total cholesterol. It will be seen that in those with normal REC concentrations most of the cholesterol extracted came from around the starting point. When the REC concentration was very high most of the cholesterol between the beta-globulins and starting point was removed. In 2 cases with extremely high extraction values, no demonstrable cholesterol was left in this area.

Discussion. Previous work has shown that lyophilization, even when followed by cold chloroform extraction, caused no demonstrable change in the migration of the main serum proteins as determined by the bromphenol blue technic(6). The beta-lipoproteins apparently constitute such a small portion of the beta-globulins that a change in their rate of migration is not demonstrated by the bromphenol blue technic. Hunter(7) found no bromphenol blue staining in the area giving the strongest staining with osmium tetraoxide vapors. The ratio of the cholesterol present in the albumin + alpha-globulin area to that present in the remainder of the strip in the 6 normal sera was the same as found by Oliver and Boyd(8) in normal subjects, namely, 28:72. The ratio for all subjects with elevated REC values was invariably less than this.

The fact that the cold chloroform usually removed little cholesterol from the albumin + alpha$_1$- and alpha$_2$-globulin areas can probably be explained by the greater stability of alpha-lipoproteins. Gurd, *et al.*(9) showed that alpha-lipoproteins are stable to lyophilization but purified beta-lipoproteins are not. Since the lyophilized serum in all cases still contained lipoproteins which migrated in the general area of the beta-globulins it would appear that some of the beta-lipoproteins are not denatured by lyophilization. The higher the REC value the greater was the percentage of total cholesterol which remained around the starting point. Swahn(10) has shown

TABLE I. Effect of Lyophilization on Electrophoretic Migration of Serum Cholesterol.

Specimen	Group & No. of subjects	% of total cholesterol in sections 1, 2	3	4	5	6, 7	REC* (range), mg %	Total chol. (range), mg %
Sera from young normal male subjects	1 (6)	21.7 (16.0–31.4)†	6.1 (4.4– 8.2)	37.8 (26.8–52.6)	29.3 (14.6–40.8)	5.0 (1.1–10.3)	16– 35	179–275
Same lyophilized		20.3 (16.2–22.8)	5.7 (2.0–11.6)	20.1 (6.2–27.6)	35.5 (24.5–43.6)	18.3 (13.6–25.1)		
Sera from patients with normal REC & total chol.	2 (7)	14.7 (10.8–14.6)	8.2 (2.8–17.0)	48.1 (40.4–51.7)	22.4 (11.8–33.3)	6.7 (4.7–10.3)	15– 35	141–291
Same lyophilized		13.8 (9.0–20.9)	6.4 (5.8– 7.0)	22.8 (14.1–29.1)	30.9 (19.4–39.7)	26.0 (17.5–33.1)		
Sera from patients with elevated REC & normal total chol.	3 (3)	8.3 (5.9– 9.8)	8.3 (2.7–14.1)	52.4 (51.5–54.1)	25.7 (23.7–28.7)	5.3 (4.7– 5.8)	141–180	256–269
Same lyophilized		8.6 (7.9– 9.2)	4.9 (4.0– 6.5)	22.2 (19.2–24.2)	31.9 (30.8–33.1)	32.3 (29.1–35.0)		
Sera from patients with elevated REC & total chol.	4 (4)	10.7 (7.7–11.2)	3.6 (2.0– 4.8)	46.5 (37.5–56.5)	32.6 (26.8–41.1)	6.7 (3.4–11.2)	107–240	353–430
Same lyophilized		9.6 (8.8–10.7)	3.2 (2.6– 3.7)	11.4 (7.7–16.0)	38.5 (33.8–43.5)	37.3 (36.9–40.7)		
Serum from patient with markedly elevated REC & total chol.	5 (1)	3.5	10.6	64.5	15.9	5.5	670	670
Same lyophilized		4.6	2.8	8.1	12.3	72.1		

* Readily extractable cholesterol—that which cold chloroform extracts from lyophilized serum. † Range in parentheses.

TABLE II. Cholesterol Distribution after Electrophoresis of Lyophilized Sera before and after Chloroform Extraction.

Sample source	Group & No. of subjects	Sections 1, 2, 3	4	5	6, 7	Sum of section values	REC* (range)
		mg %					
Normal subjects	1 (5)	50 (30–66)‡	36 (14– 62)	72 (44–101)	39 (25– 61)	197	16– 35
Residue†		49 (37–64)	30 (18– 37)	72 (57– 88)	22 (10– 34)	173	
Patients with normal REC & total chol.	2 (7)	45 (33–55)	51 (32– 74)	71 (41–109)	58 (29– 76)	225	20– 36
Residue		43 (22–50)	45 (27– 56)	61 (39– 92)	41 (22– 67)	190	
Patients with moderately elevated REC	3 (7)	45 (27–61)	62 (29–101)	113 (85–151)	94 (66–130)	314	66–141
Residue		48 (33–69)	52 (38– 74)	76 (53– 89)	55 (26– 80)	231	
Patients with markedly elevated REC & total chol.	4 (5)	55 (42–90)	74 (48–117)	135 (96–185)	250 (141–451)	514	240–833
Residue		36 (26–47)	25 (7– 55)	26 (5– 53)	17 (0– 51)	104	

* Readily extractable cholesterol—that which cold chloroform extracts from lyophilized serum. † Lyophilized sera after extraction with cold chloroform. ‡ Range in parentheses.

that in paper electrophoresis, chylomicrons remained around the starting point while lipomicrons migrated with the beta-globulins. Since lipomicrons would probably be denatured by lyophilization it is possible that the increase in the cholesterol remaining around the starting point in the lyophilized sera may represent denatured lipomicrons.

The results which have been presented suggest that the cholesterol which is extracted by cold chloroform from lyophilized serum is primarily that present as a constituent of lipo- and chylomicrons. Additional work, however, is necessary to establish this. It is interesting that Albrink, Man and Peters(11) found that the neutral fat which was removed by centrifugation at 18,000 rpm for 1 hour from clear and lactescent sera having total cholesterol values above 300 mg per 100 ml varied from 16.8 to 478 mEq per liter while the amount remaining in the subnatant varied only from 10 to 34 mEq per liter. They assume that the lipids which rose to the surface ranged from 0.1 μ in diameter, the size just sufficient to scatter light(12) to 1 μ, the size of larger chylomicrons. Any cholesterol associated with this material undoubtedly would be included in our REC fraction.

Summary. Lyophilization of serum has been shown markedly to retard electrophoretic migration of some of the cholesterol normally associated with the beta-globulin area but to exert little effect on the cholesterol associated with the albumin + alpha$_1$-globulins. The cholesterol which is extracted by cold chloroform from lyophilized serum comes primarily from that fraction normally found in the beta-globulin area or which remained around the starting point. It is suggested that the cholesterol thus removed represents primarily cholesterol which is associated with lipo- and chylomicrons.

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Coagulation of Salivary Mucoid by Freezing and Thawing of Saliva.* (22329)

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Salivary mucoid,† especially when freshly obtained, may be readily precipitated in the form of a coagulum by the addition of dilute acid to the mucoid-containing fluid(2-4). This characteristic has been used to identify and to prepare mucoid of salivary gland origin(2-5).

It has now been observed that freezing of chemically untreated mucoid-containing salivary secretions for varied periods of time results in the formation of a persisting coagulum when the secretion is thawed. This coagulum, or clot, generally resembles in appearance the mucin clot formed in response to the addition of acid. Evidence from viscosity measurements, response to dilute acetic acid, and analyses of "sialic acid" indicates that the coagulum formed after freezing and thawing is derived from the salivary mucoid.

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† The terminology suggested by Meyer(1) is used throughout this report.

Methods. Total saliva and the separate salivary secretions were collected by procedures described previously(6,7). *Chemical Methods.* The mucin clot test for the presence of polymerized mucoid was performed by adding 2 volumes of 3% acetic acid to one volume of saliva. "Sialic acid", which appears to represent the principal constituent of the carbohydrate moiety of the mucoid(4), was detected by the direct Ehrlich reaction according to the method of Werner and Odin (8). Since for this investigation only the direction of change in "sialic acid" concentration resulting from removal of the clot following freeze-thaw treatment is of importance, only relative concentrations of "sialic acid" (in terms of optical densities measured at 5650 Å in the Coleman spectrophotometer) are indicated. For amylase determinations, salivary samples were diluted 1:1000 with .025 *N* sodium chloride buffered to pH 6.9 with .01 *M* sodium phosphate. The reaction mixture consisted of 1.0 ml buffered .025 *N* sodium chloride, 1.0 ml stock soluble starch solution(9), and 0.5 ml diluted saliva. Digestion was for 15 minutes at 37.0°C. Enzyme activity is expressed in terms of milligrams of reducing sugar x 10 formed from 10 mg of substrate under these conditions. The method of Myers, Free, and Rosinski(9) was used for determination of reducing sugar. *Physical methods.* Freezing of saliva samples was accomplished either by immersion of a pyrex container in a dry ice-acetone mixture at –60 to –70°C or by placing at –10°C in the freezing compartment of a refrigerator. In each case the method and duration of freezing are stated. Thawing took place at room temperature (22-25°C) in all cases. Saliva it not a true Newtonian fluid; consequently the rate of flow of salivary fluid through a narrow bore tube in response to a difference in pressure may, to some degree, reflect the influence of physical characteristics other than viscosity. Nonetheless, it is generally true that this resistance to flow results principally from the presence of polymerized salivary mucoid and decreases when the mucoid is depolymerized(10,11) or removed as by acid clotting. With the reservations imposed by the nature of the salivary fluid clearly recognized, therefore, viscosity measurements may be used to reflect grossly the concentration of polymerized salivary mucoid(11,12). In this investigation, viscosity measurements were made using graduated capillary pipettes held vertical in matched test tubes containing 2 ml of salivary fluid. The pipettes were calibrated by observing emptying times for a 40% sucrose solution.

Results. The appearance of a persisting clot in fresh samples of frozen and thawed, but otherwise untreated, total mixed, submaxillary and sublingual salivas has been observed using more than 200 secretion samples from at least 40 individual subjects. The clot appears in the mucoid-containing secretions within 2 minutes if dry ice is employed for the freezing stage. That the clot formed after thawing is derived from the mucoid is indicated by observations resulting from 3 types of experiments. In the first type, dilute acetic acid was added to an untreated aliquot of a submaxillary secretion sample and to the supernatant fluid of a frozen-thawed aliquot at the same time after collection of the sample. Fig. 1 shows clearly the mucin clot which formed in the control and that no clot or precipitate appeared in the supernatant of the frozen-thawed aliquot.

The viscosity of a sample of submaxillary saliva standing at room temperature (25°C) may decrease by 50% within 40 minutes after collection (Table I). This effect has been attributed to nonenzymatic(10) and enzymatic(13) depolymerization processes. Freezing and thawing of an aliquot of the submaxillary saliva sample followed by removal of the resulting clot leads to an even more marked reduction of viscosity than is observed for the control aliquot within the same time

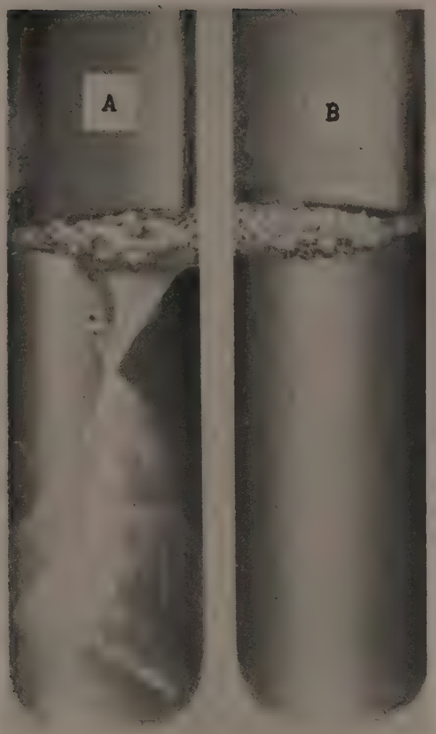

FIG. 1. Effect of addition of dilute acetic acid to an untreated aliquot (A) and to supernatant fluid of a frozen-thawed aliquot (B) of a submaxillary secretion sample 40 min. after collection. Note absence of acid-induced clot in (B), indicating decrease in polymerized mucoid in supernatant after freezing at –60°C for 2 min. and subsequent thawing at 25°C.

period after collection and indicates significant added loss of polymerized mucoid from solution as a result of the freeze-thaw treatment (Table I).

A third line of investigation which indicates that the clot which appears following thawing is derived from mucoid consists of comparing "sialic acid" values of sample aliquots which are untreated and those from which the clot appearing after freezing and thawing has been removed. "Sialic acid" is a major constituent of submaxillary mucoid and represents 25-30% of its dry weight(4). As shown by the data given in Table II, a marked decrease in "sialic acid" occurs in those aliquots submitted to freezing and thawing followed by removal of the resulting clot (Table II).

TABLE I. Effect of Freezing and Thawing on Viscosity of Submaxillary Secretion Samples.

	Sample I	Sample II
	Centipoises	
Initial viscosity	9.2	13.4
Final viscosity* (no freeze-thaw)	6.7	6.6
Final viscosity* (after freeze-thaw†)	2.4	1.8

* Final viscosity was determined 40 min. after initial viscosity determination. Except for period of freezing (where indicated), aliquots were kept at 25°C.

† Frozen by immersion in dry ice-acetone mixture at –60°C for approximately 2 min., thawed at 25°C, centrifuged, and clot discarded.

It may be noted also that clot formation after freezing and thawing is not observed with parotid secretion samples. The "sialic acid" analysis for a parotid secretion sample noted in Table II suggests the absence of "sialic acid" from this secretion. These observations are consistent and further indicate that the clot resulting from freezing and thawing of saliva samples is derived from the salivary mucoid.

TABLE II. Effect of Freezing and Thawing on Relative Concentration of Sialic Acid in Salivary Secretion Fluids.

Subject	Sample	Aliquot I (untreated)*	Aliquot II (frozen-thawed)
		Optical density × 10^2	
HF†	Submaxillary	14	5
	"	14	5
FB‡	Parotid	0	
	Sublingual	34	
	Submaxillary	17	7
LS§	"	15	3
LL§	"	43	7

* Untreated aliquots kept at 25°C before analysis.

† Aliquots I and II were submitted to analysis simultaneously 40 min. after collection of sample. Before analysis, Aliquots II were frozen 2 min. in dry ice-acetone mixture, thawed at room temperature, centrifuged and the clots discarded.

‡ Aliquots I were submitted to analysis within 30 min. after sample collection. Aliquot II (submaxillary sample) was submitted to analysis after freezing for 1 hr at –10°C and thawing at room temperature.

§ Aliquot I was submitted to analysis immediately after collection of the sample. Aliquot II was frozen 24 hr at –10°C and thawed at room temperature before analysis.

The extent to which the concentration of substances, other than mucoid, may be modified in the salivary fluid by inclusion in the coagulum has been estimated at this time only for the protein enzyme amylase. The data in Table III indicate that the amylase concentration of the salivary fluid is not appreciably modified either by the freezing and thawing procedure or by removal of the clot resulting from this procedure (Table III).

TABLE III. Effect of Freezing and Thawing of Saliva Samples on Their Amylase Activity, 5 subjects.

	Amylase activity*			
	Unfrozen aliquot		Frozen-thawed aliquot	
Secretion	Immed. after collection	After 24 hr at 4°C	Clot removed	Clot resuspended
Submaxillary	2.2	2.2	2.3	2.5
Parotid	7.9	7.9	7.9	
Submaxillary	6.8	6.4	6.2	5.4
Parotid	13.9	12.8	12.3	
Submaxillary			3.8	4.7
Total mixed			2.7	2.7
Idem			2.8	3.1

* Conditions: Amylase activity is expressed as mg reducing sugar × 10 formed at 37°C from 10 mg of starch by the action of .5 ml 1:1000 saliva. Freezing (where indicated) was for 24 hr at −10°C in refrigerator; thawing occurred at room temperature (22-25°C).

Discussion. Gore has reported that stimulated total or mandibular (mixed submaxillary-sublingual) saliva shows some opacity at collection and that on standing at 37°C for one hour appreciable precipitate, presumably of mucoid material, appears and settles out (14-16). This effect may possibly be ascribed to the action of a contaminating mucinase (12,13). The separate submaxillary secretion obtained for the experiments reported here not only has shown no opacity on collection but generally shows only slight turbidity even after standing for two hours at 25 or 37°C. For the clear delineation of the freeze-thaw effect, therefore, separate submaxillary secretion has generally been used.

The observation that a persisting clot appears after thawing of frozen fresh, mucoid-containing saliva samples and the experimental data which show clearly that this clot is derived from salivary mucoid may have application from several points of view. First, it is customary and convenient to freeze tissue or fluid samples for storage before analysis. In the case of saliva samples, care must be taken to determine that such treatment does not alter the composition relative to the analysis in question. Second, freezing and thawing may provide a convenient method for reducing the viscosity of the mucus gland secretions especially in cases where this does not alter the concentration of other components. In addition, the freeze-thaw method may prove useful as a gentle procedure for the preparation of mucoid. This method has been used successfully for the preparation of fibrinogen of extreme purity(17). The purity of salivary mucoid separated by freezing and thawing has not been determined, particularly because of difficulties involved in establishing adequate criteria of purity(1). Finally, a difference in physico-chemical properties of salivary mucoid and other mucus secretions is indicated, since Ronkin(18) has reported no change in properties after thawing frozen hyperbranchial mucus secretion from the marine snail, *Busycon*.

Summary. 1. Freezing of chemically untreated mucoid-containing salivary secretions results in the formation of a persisting clot when the secretion is thawed. This clot resembles in appearance the mucin clot formed in response to the addition of acid. 2. Evidence from viscosity measurements, response to addition of weak acid, and "sialic acid" analyses indicates that the coagulum formed after freezing and thawing is derived from the salivary mucoid. 3. It is suggested that this effect be taken into account if storage of saliva samples in a frozen state is considered.

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Serum Glutamic Pyruvic Transaminase in Cardiac and Hepatic Disease.* (22330)

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Characteristic alterations of serum glutamic oxaloacetic transaminase (SGO-T) activity have been shown to be useful in assessing the presence and degree of heart muscle cell and hepatocellular damage(1,2). The activities of glutamic oxaloacetic (GO-T) transaminase and of glutamic pyruvic transaminase (GP-T) in different tissue homogenates are compared in Table I. The activity of GP-T was found to be relatively greater in liver than other tissues as compared with GO-T activity. This observation suggested that serum glutamic pyruvic transaminase (SGP-T) might be a more specific index of liver cell damage than SGO-T because of its selective concentration in liver tissue. Furthermore, since the cardiac tissue activity of GP-T is low, myocardial necrosis might not be associated with significant alterations of SGP-T activity, thus providing a more specific serum enzyme indicator of hepatocellular damage.

Method of study. The SGP-T activity was measured spectrophotometrically. The transamination reaction (Reaction 1) is coupled to the reduction of pyruvate to lactate by reduced diphosphopyridinenucleotide (DPNH), in the presence of added excess of purified lactic dehydrogenase (Reaction 2).

$$\begin{array}{l}\alpha\text{-keto glutarate}\\ +\text{ l-alanine}\end{array} \xrightarrow{\text{SGP-T}} \begin{array}{l}\text{glutamate}\\ +\text{ pyruvate}\end{array} \qquad \text{(Reaction 1)}$$

$$\begin{array}{l}\text{pyruvate}\\ +\text{ DPNH}\end{array} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \begin{array}{l}\text{lactate}\\ +\text{ DPN}\end{array} \qquad (\quad '' \quad 2)$$

Oxidation of DPNH, and thereby the transamination reaction, is followed by measuring the decrease in light absorption at wavelength 340 mμ at which reduced diphosphopyridinenucleotide has an absorption peak. The *reagents* include: alanine, α-keto-glutaric acid, reduced diphosphopyridinenucleotide, and purified lactic dehydrogenase which can be obtained commercially. From 0.1 to 1.0 ml of serum, 1.0 ml of 0.1 M phosphate buffer (pH 7.4), 0.5 ml of 0.2 M l-alanine in buffer (pH 7.4), 0.2 ml of DPNH (1 mg/ml) and 0.1 ml of a solution of purified lactic dehydrogenase (8000 units/1.0 ml) were mixed and brought to final volume of 2.8 ml in a cuvette having a 1 cm light path. The blank contained all reactants listed except DPNH. After 15 minutes, 0.2 ml of 0.1 M α-ketoglutarate in buffer, pH 7.4, was added. The optical density at wavelength 340 mμ was

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TABLE I. Comparison of Glutamic Oxaloacetic Transaminase and Glutamic Pyruvic Transaminase in Normal Adult Tissue Homogenates.

Tissue	GO-T Units/g wet tissue (× 1000)	GP-T
Heart	156	7.1
Liver	142	44
Skeletal muscle	99	4.8
Kidney	91	19
Pancreas	28	2
Spleen	14	1.2
Lung	10	.7
Serum	.02	.016

followed for 5 minutes and the rate of decrease in optical density taken as measure of the glutamic pyruvic transaminase activity of the serum. The reaction was followed in a Beckman DU spectrophotometer at room temperature (23°C). Serum activity is expressed in units/ml/minute. One unit equals a decrease in optical density of 0.001 mμ under the conditions described.

Results. Glutamic pyruvic transaminase was measured at 23°C in sera of 260 normal humans and the activities had a mean value of 16 ± 9 units/ml/minute. When the mean value of SGP-T activity is converted to micromoles/ml hour the values fall within the range of 0.23 to 0.82 micromoles/ml/hour found by quantitative paper chromatographic assay previously reported(3).

Unless hepatic disease is simultaneously present, SGP-T appears not to be significantly altered above the normal range of activity by infectious, neoplastic, reactive, degenerative, congenital or allergic disease states. The SGP-T fails to increase significantly following necrosis resulting from acute myocardial infarction. This is probably accounted for by the fact that GP-T activity is low in heart muscle in contrast to the high activity of GO-T in cardiac tissue. Table II lists the SGO-T and SGP-T activities observed in patients with acute and chronic hepatic and cardiac disease states. From these observations and studies of 40 patients with various acute hepatic diseases it appears that acute hepatitis is associated with a rise in SGP-T activity of from 2 to 1000 times normal. The peak rise during the course of clinical hepatitis is roughly proportional to the severity of the disease process. In acute hepatitis the SGP-T activity was greater than that of SGO-T although the curves are closely parallel. The same similarity of SGP-T and SGO-T concentration was seen in patients with extrahepatic biliary obstruction. Cirrhosis in relapse is associated with increased SGO-T and SGP-T activity but the SGO-T activity is usually greater than the changes in SGP-T. Infiltration of liver by metastatic cancer, leukemic or lymphomatous disease may alter the SGO-T but has not been seen to cause elevation of the SGP-T above the normal range. The SGP-T activity has not been found to increase significantly following acute myocardial infarction in 8 patients, nor in 15 patients with other forms of heart disease. The increase in SGO-T activity in acute hepatic disease has been suggested to result from the release of liver-cell glutamic oxaloacetic transaminase into the blood stream from damaged liver cells(4,5). The presence in hepatic tissue homogenates of considerably less glutamic pyruvic transaminase activity than glutamic oxaloacetic transaminase suggests that the relatively greater increment of SGP-T activity in acute liver disease could not be solely due to release of enzyme from necrotic hepatic tissue. Variations in the release, destruction or excretion of the two enzymes or an unknown metabolic aberration are probably important contributory mechanisms and have yet to be clarified. The increase in SGO-

TABLE II. Comparison of SGO-T and SGP-T in 11 Patients with Hepatic and Cardiac Disease.

Diagnosis		SGO-T Units/ml	SGP-T
Normal individuals	Range	8-40	5-35
	Mean	20	16
Acute infectious hepatitis		330	720
Idem		[illegible]	[illegible]
Acute infectious mononucleosis hepatitis		82	120
Acute extrahepatic obstructive jaundice		108	224
Cirrhosis		134	79
Hodgkins disease of liver		43	16
Acquired hemolytic jaundice		35	24
Acute transmural myocardial infarction		61	18
Idem		228	32
Acute subendocardial infarction		56	15
Angina pectoris		35	20

T following acute myocardial infarction would appear to be primarily due to release of enzyme from necrotic cardiac muscle(6). The failure of significant elevation of SGP-T is probably due to the relatively small and therefore inconsequential amount of glutamic pyruvic transaminase escaping from damaged heart cells which when diluted in the extracellular compartment would fail appreciably to increase the SGP-T.

Summary. The measurement of SGP-T alterations has been found to be a useful tool in the diagnosis and study of acute hepatic disease and appears to be more sensitive than SGO-T in depicting acute hepatocellular damage. SGP-T unlike SGO-T has the added advantage of not being appreciably altered by acute cardiac necrosis. These preliminary conclusions require further study for confirmation.

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Vitamin A Content of the Livers of Suckling Pigs.* (22331)

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The liver of the normal newly born rat was reported by Guerrant(1) to contain very little vit. A but that the concentration more than doubled during the first week of postnatal life and then remained at a relatively constant level during the remainder of the nursing period. Braude *et al.*(2) reported liver vit. A reserves to be low in the pig at birth with the amount still decreasing during suckling if the sows were fed a vit. A deficient ration.

This paper reports the levels of vit. A found in the livers of pigs which suckled their dams for varying lengths of time.

Methods. This investigation was conducted in the summer. Thirty-five baby pigs from 6 cross-bred litters were used. One pig from each litter was sacrificed at weekly intervals until the litter was exhausted, the first group of 6 being sacrificed before they were 24 hours old. The mothers of the pigs were fed well-fortified rations containing sun-cured alfalfa hay throughout gestation and lactation. The pigs' only feed was the milk of their dam.

TABLE I. Average Vitamin A Content of Livers of Suckling Pigs.

Age at sacrifice, days	No. of pigs	Body wt, lb	Liver wt, g	Liver fat, %	Liver vit. A Per g (fresh), μg	Liver vit. A Total, μg
1	6	3.2 ± .5*	38 ± 7.9	3.4 ± .4	15.1 ± 2.6	570 ± 152
7	6	5.5 ± .9	76 ± 7.5	3.3 ± .5	30.6 ± 9.3	2281 ± 456
14	6	8.0 ± 1.9	104 ± 27.7	3.3 ± .5	26.4 ± 5.7	2689 ± 528
21	5	10.2 ± .6	135 ± 19.7	3.6 ± .6	24.4 ± 9.0	3184 ± 640
28	5	13.8 ± 3.9	179 ± 45.5	3.6 ± .4	21.7 ± 5.5	3889 ± 1622
35	5	18.5 ± 2.8	235 ± 49.4	3.3 ± .3	19.3 ± 3.4	4539 ± 975
42	2	16.9 ± 1.2	—	3.8 ± .8	17.4 ± 2.0	—

* Stand. dev. of single observation.

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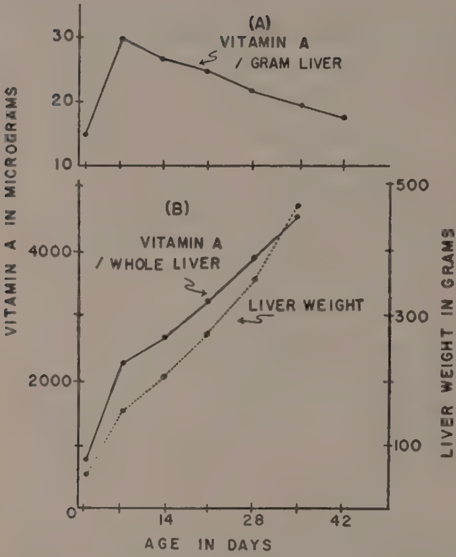

FIG. 1. Average vit. A contents in liver of suckling pigs.

The sows were fed separately from their litters and solid food was never found in the stomachs of the sacrificed pigs. The number of livers tested at each age is shown in Table I. Livers were weighed and frozen immediately after sacrifice of the pigs. Fat was extracted with ether. Vit. A was determined by the method of Gallup and Hoefer(3).

Results. Summaries of body weights, liver weights, percent fat in liver and liver vit. A according to age of pigs are presented in Table I. At one day of age, the concentration of vit. A was 15.1 μg per g of liver, while the hepatic stores for the grown-up pigs, as determined in the same season, was several times higher. The relatively low reserve of this vitamin in the liver at birth agrees well with other species(4). By the 7th day this low reserve of vit. A was doubled per unit weight and increased 4 times (570 to 2881 μg) for the whole liver while the average liver weight increased from 38 to 76 g. This increase in vit. A in the pig seems to be accounted for by the finding of Braude *et al.* (5) that sow colostrum is richer than normal milk in this vitamin.

After reaching a peak at 7 days, the concentration of vit. A in the liver decreased gradually and uniformly from about 31 to 19 μg per g of liver at 35 days after parturition (Fig. 1A). The livers grew in size with age, however, so that the total liver vit. A made a relatively steady increase from birth until the pigs were 35 days old reaching a maximum of about 4500 μg (Fig. 1B).

Summary. Livers of 35 pigs from 6 different litters were examined for vit. A content. The liver vit. A concentration increased from 15 μg at 1 day to 31 μg per g at 7 days after parturition. The average total liver vit. A increased rather uniformly from 570 μg at birth to 4539 μg at 35 days after birth.

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Propagation of Frog Renal Carcinoma in Embryonated Hens' Eggs.* (22332)

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The frog renal carcinoma (Lucké) is readily propagated in tissue culture and by intraocular implantation in frogs; homo- and heterotransplants can be made(1). It can also be grown by implantation of tadpoles (2). In the salamander the character of the tumor is reported to be altered(3). Propagation of the tumor in embryonated hens' eggs was attempted in the present study.

Materials and methods. The tumors used were from experimental frogs kindly sent by Dr. B. Lucké from Philadelphia. Naturally occurring renal tumors were also obtained from *R. pipiens* received from J. M. Hazen Co., Alburg, Vt. Embryonated eggs (White Leghorn) 8 to 12 days of age were obtained from local commercial dealers. Minced tumor tissue was placed on the dropped chorio-allantoic membrane. The window previously cut in the shell was closed and sealed with paraffin. After incubation at 35°C for 8 to 12 days the eggs were opened and the membranes were examined for growth of tumor. Histologic examination of the implanted tumor tissue and membrane was made periodically. Egg passaged tumor tissue was tested for host stability by intraocular implantation of frogs.

Results. In one experiment tumor was carried through 13 serial passages. Tumors appeared as discrete growths on the membrane and attained a size of 2-5 mm diameter. Sections of the chorio-allantoic membrane showed infiltration of the mesoderm by the tumor either in the form of cystic, papillary growths or as loosely infiltrated nests of cells. The series of passages was terminated by failure of the tumor to grow. In other attempts tumor was passed through 4 passages on 3 different occasions and through 5 and 6 passages in separate experiments. It was felt that eventual failure to grow might be associated with a tendency of the implanted tissue to become desiccated before it could become established. An attempt to avoid this difficulty was made by imbedding minced tumor fragments in chick embryo blood or adult chicken plasma clots before implantation to the egg membrane. A few drops of modified amphibian Ringer's solution (containing phosphate buffer and antibiotics) also was sometimes added onto the tissue immediately after implantation. This addition was not essential since equally good growth was obtained without it. By use of the clot technic tumor was carried through 20 serial passages. Although some tumors were as large as 10 mm in diameter the average size was 2-3 mm. Egg passaged tumor could be stored, minced in modified amphibian Ringer's solution at 4-6°C for 2 to 10 days between transfers.

Two of 4 frogs given ocular implants of tumor tissue from the 20th egg passage developed local tumor growths and had kidney tumors when examined 2 to 6 months after inoculation. Attempts to grow egg passaged tumor in the yolk sac of 5-day embryos failed. Microscopic examination of sections of the yolk sac made 13 days after implantation revealed only few, necrotic tumor cells.

Frog tumor grown at 36-37°C in roller tube, tissue cultures containing chicken plasma clot, and modified amphibian Ringer's solution grew readily in eggs after culture, *in vitro*, for 3 to 5 weeks(4).

Discussion. Heterologous transplants of amphibian tumor tissue to hens' eggs grew irregularly in serial passages. Vigorous growth of tumor in one passage might be followed by poor growth or failure to grow in the next passage or *vice versa.* It is not clear to what extent this is due to failure of the tissue to establish contact with the chorio-allantoic membrane suitable to permit nourishment of the tumor. It seems unlikely that the relatively high environmental temperature is in-

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hibitory since the tissue, *in vitro,* survived a temperature of 36-37°C for a prolonged period. The relatively slow rate of growth of the tumor tissue possibly contributes to the difficulty of implantation on the membrane. There was no evidence of alteration in the type of growth of tumor tissue during the course of 20 heterotransplants, such as was noted elsewhere during passage of frog renal carcinoma in newts(3) and during passage of a murine carcinoma in eggs(5).

Summary. The frog renal carcinoma (Lucké) has been propagated on the chorio-allantoic membrane of hens' eggs for 20 serial passages. Growth was irregular.

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Glycogen Stores in Glucagon-Treated Rats. II. Effect of Alloxan Diabetes.* (22333)

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Chronic treatment with small doses of glucagon causes no change in liver glycogen(1), while large doses may cause a significant increase(2). Further study of this seemingly paradoxic effect of the "glycogenolytic" hormone demonstrated that, although single intraperitoneal injections of large doses of glucagon into normal rats cause an immediate decrease in liver glycogen, this is followed 24 hours later by a marked increase above normal levels(3). When the animals are sacrificed 24 hours after the last glucagon injection, as is done in chronic experiments, the short-lived glycogenolytic effects are no longer detectable because glycogen has been resynthesized. This resynthesis of glycogen may be due to 3 causes: 1) reversal of the phosphorylase reaction(3); 2) increased secretion of adrenal cortical hormones, in a manner similar to that which follows the injection of epinephrine(4); or 3) increased secretion of insulin, as a result of glucagon hyperglycemia (5). The purpose of this work was to investigate the third hypothesis by studying the effect of glucagon and epinephrine on liver glycogen, muscle glycogen and adrenal ascorbic acid in alloxan-diabetic rats.

Materials and methods. Seventy-two adult Sprague-Dawley male albino rats were used. The animals were kept on a diet of Purina checkers *ad libitum* and fasted for 24 hours before the experiments. Alloxan diabetes was produced according to the method of Sturtevant(6). Since glycogen deposits of alloxanized animals vary with the severity of the diabetes(7-9), only rats with established diabetes of more than 6 weeks' duration and with severe (4+) glycosuria and polyuria were used.‖ Glucagon (Eli Lilly and Co., lot no. 208-158B-197; 2.5 or 5.0 mg/kg),¶ epinephrine (0.15 mg/kg) and glucose (1 g/kg) were injected intraperitoneally. After 2 or 24 hours, the animals were decapitated, samples of liver and of gastrocnemius muscle and both

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‖ Alloxan diabetic rats were a gift of Dr. F. M. Sturtevant of G. D. Searle and Co.

¶ Glucagon was a gift of Dr. O. K. Behrens of the Lilly Research Laboratories.

TABLE I. Effect of Glucagon and of Epinephrine on Liver and Muscle Glycogen and on Adrenal Ascorbic Acid of Alloxan-Diabetic Rats. mg/100 g of fresh tissue. Fasting time 24 hr; glucose 1 g/kg.

No. of rats	Time after inj. (hr)	Glucagon (mg/kg)	Epinephrine (mg/kg)	Glycogen Liver Avg SE_m	Glycogen Liver % change	Glycogen Muscle Avg SE_m	Glycogen Muscle % change	Adrenal ascorbic acid Avg SE_m	Adrenal ascorbic acid % change
5	2	—	—	574 ± 74		387 ± 42		380 ± 24	
5		5	—	102 ± 11	−82*	316 ± 60	−19	306 ± 21	−20
4	2	—	—	—	—	310 ± 47		403 ± 32	
6		5	—	—	—	559 ± 10	+80*	290 ± 15	−28*
5	24	—	—	932 ± 67		413 ± 24		365 ± 44	
5		5	—	1045 ± 94	+12	319 ± 35	−23	321 ± 16	−12
5	24	—	—	1348 ±159		—	—	434 ± 21	
5		5	—	1140 ±101	−16	—	—	411 ± 26	− 5
5	24	—	—	639 ± 84		307 ± 45		326 ± 18	
5		5	—	544 ± 95	−16	383 ± 55	+24	302 ± 26	− 7
4	⅓	—	—	1811 ±162		212 ± 47		270 ± 34	
6		—	0.15	1356 ± 84	−25	114 ± 11	−46	211 ± 22	−22
6	⅓	—	—	4180 ±154		319 ± 25		368 ± 15	
6		—	0.15	2165 ±190	−49*	205 ± 28	−36*	267 ± 14	−27*

* $P < 0.05$ or better.

adrenal glands were removed as rapidly as possible. Liver and muscle were placed in tared centrifuge tubes containing 30% KOH. Glycogen was determined according to the method of Good, Kramer and Somogyi(10), supplemented by the method of Nelson(11). The adrenal ascorbic acid was determined according to the method of Roe and Kuether (12). Each experiment consisted of a group of rats treated with glucagon or epinephrine and a control group treated with saline. All animals received glucose 24 hours before death. The statistical significance of the difference between mean experimental and control values was calculated according to Fisher (13).

Results. The results, presented in Table I, were as follows: 1) 2 hours after the injection of glucagon there was a marked and significant decrease in liver glycogen accompanied, in 1 experiment, by a marked and significant increase in muscle glycogen; 2) 24 hours after the injection of glucagon the liver and muscle glycogen had returned to control values; 3) in no case did liver glycogen increase above the control values; 4) no significant changes in adrenal ascorbic acid were observed after glucagon injection, except in one experiment where a significant decrease was noted; 5) twenty minutes after the injection of epinephrine there was a decrease in liver and muscle glycogen and adrenal ascorbic acid. These changes were not statistically significant; 6) 2 hours after the injection of epinephrine, the reduction in liver and muscle glycogen and adrenal ascorbic acid became more pronounced and statistically significant; 7) the average weight of the adrenal glands of alloxan diabetic animals was 38.6 mg/100 g of body weight compared to 16.3 mg/100 g in the normal controls ($P<0.01$).

Discussion. The liver and muscle glycogen content of fasted alloxan-diabetic animals is similar to that of fasted normal rats(3), indicating that well established severe alloxan diabetes does not interfere with glycogen storage. This is in accord with the observation of others(8,9,14-16). The results indicate also that alloxan diabetes does not interfere with the glycogenolytic effect of glucagon and epinephrine, but completely prevents the secondary increase in liver glycogen observed in normal animals 24 hours after the injection of glucagon and 2 hours after the injection of epinephrine. This suggests that insulin is necessary for this phenomenon. The adrenal ascorbic acid which, in the normal animal, decreases markedly following glucagon or epinephrine injections does not appear to be significantly changed in most alloxan diabetic rats. This may be due to the fact that in alloxan diabetes, a state of increased adrenal activity already exists, as suggested by the greater weight of the adrenal glands. Although hypertrophy in itself does not prove hyperfunction(17), increased adrenal cortical activity in alloxan diabetic rats has been observed also by other workers(8,18). In 1 out of 4 experiments, the injection of glucagon was followed by a significant increase in muscle glycogen, confirming previous observations in normal animals(3,19). This phenomenon appears to be contrary to the observation that glucagon inhibits the synthesis of muscle glycogen produced by insulin(20), and probably is due not to glucagon itself, but to secondary insulin or adrenal cortical secretion(3). Its occurrence in severely diabetic rats makes the first possibility unlikely. On the other hand, the decrease in adrenal ascorbic acid coincident with the increase in muscle glycogen in one experiment suggests that the phenomenon may be the result of adrenal cortical stimulation.

Summary and conclusions. 1) The effects of glucagon and of epinephrine on liver and muscle glycogen and adrenal ascorbic acid of alloxan diabetic rats were studied. 2) Twenty minutes after injection of epinephrine a decrease in liver and muscle glycogen was observed. The decrease was still greater after 2 hours. 3) Two hours after injection of glucagon there was a decrease in liver glycogen and, in one experiment, an increase in muscle glycogen. 4) Twenty-four hours after injection of glucagon, liver glycogen had returned to, but not above, the initial level. 5) The adrenal glands of alloxan diabetic rats are significantly larger than those of normal rats. The injection of glucagon or epinephrine may further stimulate adrenal cortices, as indicated by occasional decrease in their ascorbic acid content. 6) It is concluded that: a) the glycogenolytic effects of glucagon and epinephrine are unimpaired in alloxan diabetes, and b) the delayed increase in liver glycogen, observed in normal rats after injection of epinephrine or glucagon, requires the presence of an intact insulin-secreting system capable of being stimulated by epinephrine or glucagon hyperglycemia.

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Fallacy of "Crushing Death" in Frozen Bacterial Suspensions. (22334)

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It is well known that when a bacterial culture is frozen and thawed a portion of the cell population is killed. It has been generally accepted that death results from some mechanical effect produced as a result of the solidification of the suspension—either from crushing by ice crystals or from pressure resulting from ice formation(1-6). It has been demonstrated repeatedly that different species possess different susceptibilities to the lethal effect of freezing(7-9). If the lethal factor is mechanical, the differences in susceptibility to freezing must reflect differences in susceptibility to this mechanical factor. It becomes necessary, therefore, to conclude that the cells of species less susceptible to the lethal effect of freezing are physically stronger than the cells of species more susceptible. In fact, this conclusion has been presented on at least one occasion(5).

With experiments summarized in this paper we have sought to correlate the physical strength of cells with their susceptibility to freezing. In this manner, evidence for or against the ice crushing theory could be obtained. To determine the relative physical strength of different bacteria we have subjected them to violent shaking with small glass beads in the Mickle Tissue Disintegrator (MTD). Here the lethal factor is certainly mechanical, since very little death occurs in the absence of beads or abrasive, even upon prolonged shaking.

Materials and methods. Each experiment consisted of subjecting 2 different organisms to the 2 lethal conditions—successive freezings and vigorous shaking with beads. From each experiment, therefore, 4 sets of data were obtained. In every experiment *Escherichia coli* strain 69L-15 was one of the organisms employed; it was always the "reference organism" to which the behavior of a second organism was compared. The bacteria were cultivated as already outlined(9). The 2 cultures were centrifuged, the culture broth decanted, and the cells resuspended in broth of the following percentage composition: yeast extract (Balt. Biol. Lab., dehydrated), 1.0; Tween 80 (Atlas Powder Co.), 0.1; K_2HPO_4, 0.2; and $MgSO_4 \cdot 7H_2O$, 0.01. The cell concentration was adjusted (using broth) so that the viable cell count of each suspension would be approximately the same. An aliquot (7 ml) of the reference suspension (*E. coli*) was placed into a sterile vial containing 3 g of glass beads (0.057 mm average diam.).* The vial was stoppered and placed in its receptacle on one of the steel reeds of the MTD. A second aliquot (5 ml) of the suspension was placed into a sterile test tube (15 mm by 150 mm) fitted loosely with an aluminum cap. Aliquots of the "comparison suspension" were then dispensed in like manner. (Comparison suspensions were prepared using the following species: *Microbacterium flavum* strain 0J 10, *Lactobacillus fermenti* strain 69L-3, and *Bacillus pumilus* strain NRS 236.) The contents of the 2 test tubes were frozen simultaneously by placing the tubes in a −22°C alcohol bath. After 25 minutes at −22°C they were thawed simultaneously by transferring them to a +35°C water bath. A 0.1 ml sample was removed from each tube and the tubes were placed again at −22°C. In this manner, the suspensions were frozen and thawed 8 or more successive times. The 0.1 ml samples were diluted and plated in order to determine the number of cells which remained viable after a given number of successive freezings. Concurrent with the freezing-thawings, the suspensions in the MTD were being vigorously shaken. Periodically, the instrument was stopped for a time just sufficient to remove a 0.1 ml sample from each vial and to interchange the vials between the 2 receptacles. Switching the vials after each sampling was done in order to nullify the

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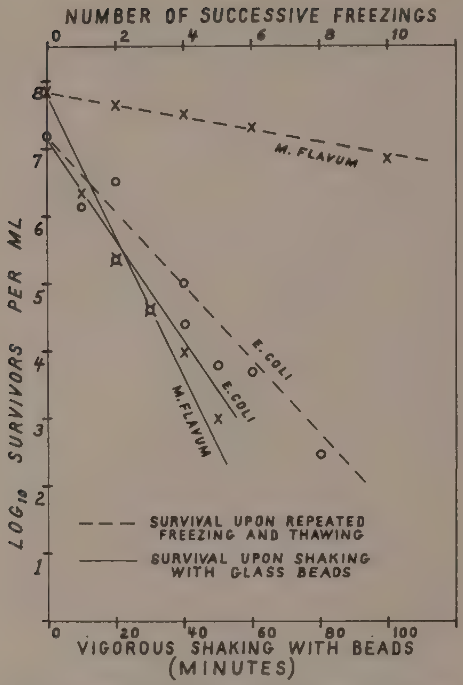

FIG. 1. Comparison of survival of *Microbacterium flavum* and *Escherichia coli* when subjected to successive freezings and when subjected to shaking with beads in the Mickle Tissue Disintegrator.

effect any small inequality in the amplitude of vibration of the 2 reeds would have upon survival of the bacteria in the respective vials. During shaking the vials were kept cool by means of a jet of CO_2. The 0.1 ml samples were diluted and plated in order to determine the number of cells which survive shaking for various periods of time. The dilution and plating technics already have been described (9,10).

Results. Fig. 1 illustrates that the susceptibility of *E. coli* to freezing is much greater than that of *M. flavum*, in spite of the fact that, as evidenced by rate of destruction in the MTD, the cells of *E. coli* appear to be physically stronger than those of *M. flavum.* In an experiment comparing *L. fermenti* with *E. coli,* little difference in the susceptibility to freezing of these 2 species was detected, although the cells of the latter species manifested a greater susceptibility to destruction in the MTD. On the other hand, in an experiment comparing *B. pumilus* with *E. coli* the cells of the former organism were less susceptible both to freezing and to shaking. There is no doubt that if sufficient pairs of species had been tested every possible combination of relative susceptibilities to the 2 lethal conditions would have been obtained. It is apparent that there is no correlation between the physical strength of cells and their susceptibility to freezing.

The susceptibility to freezing manifested by any given species is affected by both its remote and recent history. Species that are facultative toward oxygen are much less susceptible to the lethal effect of freezing when cultivated under forced aeration(9,10). In Fig. 2 it is readily apparent that cells of *E. coli* which had been aerated during growth are extremely resistant to freezing, whereas those cultivated in the customary manner show the usual susceptibility. It is important to note that the cells from the 2 cultures do

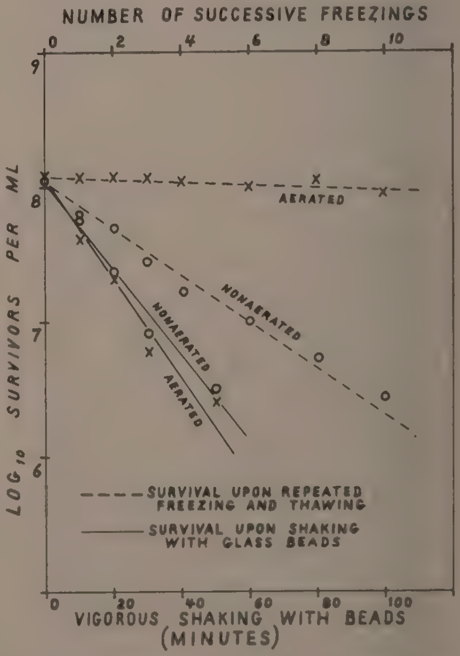

FIG. 2. Comparison of survival of aerated and nonaerated cultures of *Escherichia coli* when subjected to successive freezings and when subjected to shaking with glass beads in the Mickle Tissue Disintegrator.

not differ appreciably with respect to their physical strength. Indeed, it hardly would be expected that aeration would render cells physically stronger; rather, it might be expected that the cells would be altered in permeability and/or concentration of intracellular metabolites and enzymes. The greater resistance of aerated cells is undoubtedly due to such alterations. It is suggested that the actual cause of death upon freezing and thawing is not due to a mechanical factor but is due to some chemical or physicochemical phenomenon. The effect of solutes and initial cell concentration upon survival, as well as the very shape of the survival curves(9,10) supports this contention.

Summary. There appears to be no correlation between the physical strength of cells and their susceptibility to the lethal effect of freezing. It is therefore unlikely that the lethal factor of freezing-thawing is mechanical.

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Resistance of Specifically-Sensitized *Treponema pallidum* to Methylene Blue Stain.* (22335)

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During studies of toxoplasmosis Sabin and Feldman(1) developed a serodiagnostic test based on the observation that *Toxoplasma gondii*, which is normally stained by methylene blue, lost affinity for the dye when incubated with homologous antiserum and a thermolabile factor in normal serum. Recent experiments(2) showed that virulent *Treponema pallidum* becomes similarly resistant to methylene blue when incubated with syphilitic serum and complement. The purpose of the present studies was to explore possible applications of the reaction in the diagnosis of syphilis.

Materials and methods. The treponeme stain consisted of 0.10 g methylene blue† and 0.025 g Na_2CO_3 in distilled water to make 100 ml. It was prepared by diluting 1 ml of 2.50% Na_2CO_3 with 89 ml water, then adding rapidly 10 ml of a stock 1% solution of the dye. Both stock and dilute dye solutions were stored at 3-6°C, care being taken however to discard any showing sediment or contamination. New glass slides and coverslips, used in microscopic examination of test mixtures, were cleaned in ethanol (70%) and wiped dry with clean gauze. All other glassware was cleaned scrupulously with sulfuric-chromic acid mixture. The basic test procedure was the treponemal immobilization test(3,4). To 0.05 ml of serum in 13 x 100 mm test tube were added 0.40 ml of suspension containing 7-9 x 10^6 treponemes/ml and 0.05 ml guinea pig complement (C′); 0.05 ml of inactivated guinea pig C′ was substituted in otherwise identical serum control. The mixtures were incubated 18 hours at 35°C in an atmosphere of nitrogen (95%) and carbon dioxide (5%). The treponemes in test and control mixtures were then suspended uniformly by swirling, and samples (about 0.01 ml) transferred with capillary pipettes[‡] to opposite ends of a glass slide; delivery was effected by touching capillary tips to the slide. Two such slide preparations were made. The first served for immobilization tests, the droplets being surmounted with coverslips and given to other personnel for independent examination. An equal volume of methylene blue solution was added to the second set of droplets; individual capillaries were used for each addition and the dye and treponemes mixed with the tips. Coverslips were applied and the mixtures allowed to stand for 15 minutes at room temperature; this interval was essential for clearcut differentiation of stained and unstained treponemes. The frequency (%) of stained organisms in test and control mixtures was then determined by dark-field microscopic examination of fifty treponemes (magnification, 430 X). In this connection it should be noted that personnel familiarized with the dye and immobilization tests found the differentiation between stained and unstained organisms simpler than the decision regarding motility which was frequently complicated by sluggish or intermittently motile treponemes. The method adopted in calculating percentage of specifically dye resistant treponemes corresponded with that(4) used in determining percentage specifically immobilized; *i.e.*

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* Presented in part at Symposium on Recent Advances in the Study of Venereal Diseases, April, 1955, Washington, D.C.

† Methylene blue chloride (dye content, 87%), National Aniline Division, Allied Chemical and Dye Corp., New York City.

$$\text{Specific dye resistance } (\%) = 100\ \frac{(\%\text{ stained in control}) - (\%\text{ stained in test})}{(\%\text{ stained in control})}$$

Comparison of dye and immobilization tests was further simplified by adopting the same convention in interpretation; *i.e.*, more than 50% specific dye resistance was considered a reaction, 21-50% a weak reaction, and less than 21% no reaction. Tests for residual C′ in each serum-treponeme C′ mixture were conducted shortly after removal of samples for dye and immobilization tests; each tube containing test mixture received 0.25 ml of a suspension containing 2 x 10^8 optimally-sensitized(5) erythrocytes/ml, and was incubated 30 minutes at 37°C. Fifty to 100% hemolysis was considered an acceptable result(4); less than 50% hemolysis however indicated a degree of C′ deterioration warranting repetition of test. *Syphilitic rabbit sera* were obtained from animals infected experimentally with Nichol's strain of *T. pallidum*. Samples of representative specimens were absorbed with cardiolipin antigen floccules(6); others were absorbed with virulent treponemes, the serum-treponeme-C′ mixtures being incubated anaerobically for 18 hours at 35°C and the organisms separated by centrifugation. Human sera were obtained from patients with syphilis and from individuals without clinical or anamnestic evidence of treponematosis. All were stored at –20°C. Just prior to testing, the required volume of thawed serum was heated for 30 minutes in water bath at 56°C.

Results. The results obtained in parallel methylene blue (TPMB), immobilization (TPI), and cardiolipin flocculation (CF) tests of a syphilitic rabbit serum appear in Table I A; aliquants of the same serum dilutions were used in the 3 titrations. Essentially the same degrees of reaction were observed in the parallel TPMB and TPI tests, and complement (C′) was required in both reactions (*cf* tests and C′-free controls). Attempts to absorb the serum components responsible for dye resistance or immobilization were unsuccessful, sera treated repeatedly with 6 x 10^7 *T. pallidum* per ml remaining unchanged in reactivity. Absorption with cardiolipin antigen floccules, however, reduced the titer of lipid flocculating (CF) antibody without affecting reactivity in the TPMB or TPI tests; the results obtained in titrations of a lipid absorbed rabbit serum are given in Table I B.

The relative sensitivity and specificity of the dye test were evaluated in a serologic study of 169 selected individuals; the TPI test was performed concurrently as a control. Table II summarizes the findings. Sixty patients presenting clinical or anamnestic evidence of syphilis showed almost identical TPMB and

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[‡] Melting Point Capillary Tubes, 1.5-2.0 x 100 mm, No. 34500, Kimble Glass Division, Owens-Illinois Glass Co., Toledo, O.

TABLE I. Treponemal Immobilization (TPI), Methylene Blue Resistance (TPMB), and Cardiolipin Flocculation (CF) Tests of a Syphilitic Rabbit Serum.

	TPI test			TPMB test			CF test
	Treponemes motile, %			Treponemes stained, %			
Serum dilution	Test	Control	Specific immobilization, %*	Test	Control	Specific dye resistance, %*	Degree of reaction
A. Reactions of original serum							
1: 8	10	100	90	6	100	94	4+
1: 16	36	98	63	26	98	73	3+
1: 32	68	96	29	74	94	21	1+
1: 64	90	100	10	84	98	14	—
1:128	98	100	2	98	96	—	—
B. Reactions after absorption with cardiolipin antigen floccules							
1: 8	8	100	92	10	96	90	—†
1: 16	32	98	67	22	100	78	—
1: 32	70	94	26	74	98	25	—
1: 64	90	100	10	86	98	12	—
1:128	92	100	8	92	98	6	—

* Calculated as $100 \frac{b-a}{b}$, where a is % of stained (or motile) treponemes in the test with complement (C′), and b is that in the C′-free serum control.

† A trace (±) reaction persisted in the test with undiluted (1:1) serum.

TPI reactions, the single exception being an individual with secondary syphilis whose serum was negative in the dye test but reacted weakly in the test for immobilizing antibody. Of special interest were 12 persons whose sera were known to yield non-specific immobilization (NSI) reactions; *i.e.*, immobilization of treponemes in C′-free controls; they were retested to determine whether factors invalidating the TPI test would interfere in the test for methylene blue resistance. All 12 sera yielded non-specific (NSMB) reactions in the dye test. In the group of non-syphilitic individuals there were 19 patients with non-treponemal infections or disorders, 7 of whom reacted serologically in tests(6,7) using cardiolipin antigens. Eighteen of the 19 proved negative in both TPMB and TPI tests. One, a patient whose serum reacted with cardiolipin antigens, was negative in the TPMB but reacted weakly in the TPI test; the diagnosis in this instance was erythema nodosum. The

TABLE II. Treponemal Immobilization (TPI) and Methylene Blue Resistance (TPMB) Tests of Sera from Syphilitic and Non-Syphilitic Persons.

		Persons showing given result* in the							
		TPI test				TPMB test			
Clinical diagnosis	Persons tested	R	wr	—	NSI	R	wr	—	NSMB
Syphilis									
Primary	5			2	3			2	3
Secondary	6	2	1		3	2		1	3
Early treated	5	1		4		1		4	
Congenital	3	3				3			
Neurological	24	17	1	2	4	17	1	2	4
Cardiovascular	4	2			2	2			2
Latent (probable)	13	11	2			11	2		
Diseases other than syphilis									
Reactive in STS†	7		1	6				7	
Non-reactive in STS	12			12				12	
Healthy	90			89	1			89	1

* Designated as reaction (R), weak reaction (wr), or no reaction (—); the symbols NSI and NSMB indicate that non-specific immobilization or dye resistance was encountered both in the test with complement (C′), and in the C′-free serum control.

† Standard flocculation and/or complement fixation tests(6,7) using cardiolipin antigens.

90 healthy persons who served as controls were young men undergoing comprehensive physical and laboratory tests as candidates for a service academy. One of this group yielded non-specific reactions (NSMB and NSI); the remainder were sero-negative.

Attempts to substitute killed *T. pallidum* in the TPMB test were generally unsuccessful. A variety of treponemacidal agents were tested. Among the chemicals found unacceptable were aqueous ethanol (5%), formalin (1%), phenol (0.1%), benzalkonium chloride (0.1%), sodium hypochlorite (0.1%), sodium ethyl mercuri thiosalicylate (0.1%), sodium citrate (2%), and a polyethylene derivative of sorbitan monooleate (Tween 80, 10%). The possibility of utilizing the treponemacidal effect of methylene blue was also explored; pre-stained organisms however proved resistant to decoloration despite prolonged incubation (18 hrs at 35°C) with syphilitic serum and complement. Heating the treponemal suspension for 10 min. at 56°C frequently immobilized the organisms without altering their capacity for specific dye resistance, but the effect was not a predictable one.

Discussion. The component of syphilitic serum conferring specific resistance to methylene blue appeared to be associated if not identical with treponemal immobilizing antibody. This conclusion was suggested by the close agreement observed in quantitative dye and immobilization tests and by the common requirement for complement (Table I). It was supported by almost identical distribution of reactions in qualitative TPMB and TPI tests of patients in the various stages of syphilis (Table II). The relationship of the responsible serum component to *T. pallidum* was not demonstrable by specific absorption, an experience which has an interesting parallel in the recent failure to neutralize treponemal immobilizing antibody with soluble antigen(8). Whatever its nature, however, the essential factor in syphilitic serum was distinct from the antibody concerned in reactions with cardiolipin antigens (Table I). As such it provides an additional basis for differentiating the patient with latent syphilis from the uninfected individual whose serum reacts with lipid antigens; evidence of the usefulness of the TPMB test in this respect was obtained in the present study (Table II). Further evaluation of the test is essential, however, and continuing studies should emphasize particularly the development of a killed treponemal suspension exhibiting the specific dye resistance characteristic of live *T. pallidum*. Being based on specific alterations in the treponemal staining reaction, the TPMB test has certain advantages over one(3,4) relying on changes in motility, and the development of a stable, killed antigen would mark an important advance in standardization.

Summary. Virulent *T. pallida,* which are normally stained by methylene blue, become resistant to the dye following incubation with syphilitic serum and complement. The responsible factor in syphilitic serum is associated if not identical with treponemal immobilizing antibody. The reaction provides the basis for a specific diagnostic test for syphilis.

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Coenzyme A Activity in Tissues of Normal and Scorbutic Guinea Pigs. (22336)

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By using labelled acetate Bloch *et al.*(1-4) showed that cholesterol is formed in the body from acetate. Adrenal cholesterol diminished in scurvy(5). Scorbutic guinea pigs excreted a lesser amount of injected p-aminobenzoic acid (PABA) in the acetylated form(6). Diminished adrenal cholesterol in scorbutic animals seemed possibly due to diminished acetylation in the body. But it was also observed(6) that while cholesterol content of adrenals, spleen and lungs diminished, that of testes and small intestine increased and there was no change in the cholesterol content of liver and kidney(6). It was, therefore, not clear if the total synthesis of cholesterol by a scorbutic guinea pig was altered. Becker *et al.*(7) reported that liver and adrenals of scorbutic guinea pigs fed labelled acetate contained cholesterol with higher specific activity, which indicated that adrenal glands of scorbutic guinea pigs synthesized more cholesterol from administered acetate. It was, therefore, difficult to explain why scorbutic guinea pigs showed diminished acetylation reaction. Decreased acetylation of PABA might be due to diminished coenzyme A (Co A) activity of tissues. The present investigation was undertaken to study Co A activity of tissues of scorbutic and paired fed normal guinea pigs. Cholesterol content of the whole body of the animals was also determined.

Methods. Production of scurvy. Selection of guinea pigs and feeding a scorbutic diet, with or without the supplement of ascorbic acid by the paired feeding technic, was the same as described previously(5). *Studies with urine.* Acetylation studies were carried out as described previously(6). Results are given in Table I. *Adenosinetriphosphate* (ATP) was prepared as dibarium salt by the method of Dounce *et al.*(8) from rabbit muscle. Before use, the barium salt was converted into potassium-ATP. *Preparation and aging of apoenzyme from pigeon liver.* Apoenzyme was prepared from pigeon liver and aged by the method of Kaplan and Lipmann (9).

TABLE I. Percentage of Acetylated PABA Excreted in Urine by Guinea Pigs. Average of 3 days excretion.

Normal	Scorbutic	
25.0 ± 2.4* (5)	17.4 ± 2.5 (5)	*t*: 2.2

* Stand. error.
Figures in parentheses indicate No. of animals.

Results. Studies with tissues. Scorbutic animals were sacrificed on the 25th day of experiment along with their paired fed controls and Co A activity of liver, kidney and brain was determined by the method of Kaplan and Lipmann(9). Dry weights of the tissues were determined and Co A activity was expressed on dry weight basis. No Co A preparation of known potency was available. Crude preparation of Co A was obtained by the method of Kaplan and Lipmann(9) from rabbit liver and was used as the reference Co A. Activity-concentration curve was prepared with this reference Co A for each batch of extract of apoenzyme over a wide range including the saturation point. Half of the maximum activity was taken as unit. From this standard curve the Co A contents of the test samples were obtained directly. Results are given in Table II.

Estimation of total body cholesterol. Animals were killed, gastrointestinal tracts washed, whole body minced, weighed, dried

TABLE II. Coenzyme A Contents of Tissues of Normal and Scorbutic Guinea Pigs. Lipmann units of Co A/g dry tissue.

	Liver	Kidney	Brain
Normal (9)	959 ± 58	420 ± 35	294 ± 27
Scorbutic (9)	1007 ± 54	500 ± 40	301 ± 21

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* One of us (S.L.) received Kalyan Kumar Mukherjee Research Scholarship from Calcutta University, India.

TABLE III. Total Body Cholesterol of Normal and Scorbutic Guinea Pigs. mg/100 g fresh tissue.

Normal (8)	219 ± 5.7	Scorbutic (8)	269 ± 9.8	*t*: 4.4

at 85°C for 16 hours, powdered and extracted with acetone in a Soxhlet apparatus for 24 hours. Cholesterol was estimated in aliquots of extracts by the method of Sobel and Mayer (11). Results are given in Table III.

Discussion. Scorbutic guinea pigs showed decreased acetylation of administered PABA. Co A activity of tissues of scorbutic guinea pigs did not change. As the paired feeding technic was employed the consumption of pantothenic acid was similar in both the groups of animals. The decreased excretion of injected PABA in acetylated form by scorbutic guinea pigs indicated that ascorbic acid was in some way concerned in acetylation reactions in the body. A significant increase in the whole body cholesterol of scorbutic guinea pigs was observed. This might be due to either increased synthesis or decreased utilization of cholesterol in the body of a scorbutic guinea pig. The high rate of incorporation of acetate into cholesterol by the livers of alloxan-diabetic rats is reduced by insulin(12). Recent observations indicate that in insulin insufficiency and also in vit. C deficiency the oxidation of acetate in the tricarboxylic acid cycle is hampered(13-15). Vit. C deficiency in turn decreases the insulin content of pancreas(16). Thus acetate pool which is not burned through tricarboxylic acid cycle might be utilized in the increased formation of cholesterol. The increased cholesterogenesis in the scorbutic guinea pigs, therefore, might be due to diminished insulin synthesis.

Summary. Acute scurvy was produced in guinea pigs by paired feeding technic. Urinary excretion of injected PABA in the acetylated form was estimated in scorbutic and paired fed normal guinea pigs. Scorbutic animals excreted decreased amount of acetylated PABA. Co A activity of liver, kidney and brain of scorbutic and paired fed control guinea pigs was determined. Co A activity which was present in the tissues of normal guinea pigs did not change in scurvy. Total body cholesterol of scorbutic guinea pigs increased significantly. The implications of the results obtained have been discussed.

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In vitro Conversion of Testosterone to 17β-Hydroxyandrostan-3-one.* (22337)

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In schemes for the metabolism of testosterone, a theoretical intermediate which has been postulated but up to now not demonstrated is 17β-hydroxyandrostan-3-one(1). In this paper evidence is presented for the formation of this steroid when testosterone was incubated with a homogenate of liver from female rats.

Method. Two incubations were carried out. In both, female Sprague-Dawley rats weighing 200-250 g were decapitated, and the livers removed and homogenized immediately in a Potter homogenizer. The homogenizing medium was that described by Samuels and West (2). The incubations were carried out in a Dubnoff shaking incubator, in air, at 38°C. 1.5 ml of homogenate were added to 20 ml beakers, containing 1 mg testosterone in 0.06 ml propylene glycol, and 3 mg diphosphopyridine nucleotide (DPN) were added just before incubation.

Incubation No. 1. One gram of liver was homogenized per one ml of medium and the gauze-filtered homogenate was diluted with an equal amount of homogenizing fluid. There was approximately one-half gram of tissue per incubation flask. Twenty beakers were incubated for 90 minutes. After incubation, the reaction was stopped by addition of 0.15 ml 3 M acetate buffer, pH 5. The incubation mixtures were pooled and extracted 3 times with ethyl acetate. The ethyl acetate extract was dried over sodium sulfate, filtered, and the solvent removed *in vacuo*. Without any further purification, this extract was chromatographed on a 14 cm paper strip, in the ligroin-propylene glycol system(3) for 27 hours. A zone giving a blue Zimmermann color, having a mobility slower than that of androsterone, was eluted. This eluted material was taken up in chloroform, washed with a small volume of water, and dried over sodium sulfate. After removal of the chloroform, *in vacuo*, it was analyzed by infrared spectroscopy† and found to contain 17β-hydroxyandrostan-3-one as the major component. The crude material weighing 8 mg was rechromatographed in ligroin-propylene glycol for 24 hours, with crystalline 17β-hydroxyandrostan-3-one as standard on an adjacent strip. The mobilities of the isolated material and standard were the same. The eluted material was crystallized from acetone and methanol and melted at 175-178°C. When mixed with an authentic sample melting at 178°, 182-3° the mixture melted at 175-8°C. The melting point was depressed to 148°-167°C when mixed with androsterone melting at 181-4°C. The infrared spectrum of the isolated crystalline material was identical to that of the authentic sample.

Incubation No. 2. 13 g of liver were homogenized to a total volume of 35 ml. This homogenate contained about 0.56 g of tissue per 1.5 ml. Twenty beakers were incubated. After 2 hours incubation, 4 groups of 5 incubation mixtures were pooled, and 150 ml of acetone were added to each pool. After shaking for an hour, the acetone extracts were filtered and evaporated to dryness, *in vacuo*. The acetone extracts were dried overnight in a vacuum desiccator, over calcium chloride. The extracts were chromatographed for 20 hours in ligroin-propylene glycol. The zones whose mobilities were slower than that of androsterone were pooled and rechromatographed for 20 hours and the major Zimmermann-reacting (blue) zone was eluted for infrared analysis. The major component was identified as 17β-hydroxyandro-

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† Infrared analyses were carried out by Mr. Paul Skogstrom under the direction of Dr. Harris Rosenkrantz.

stan-3-one.

Summary and conclusion. Incubation of testosterone with an homogenate of liver from female rats led to formation of 17β-hydroxyandrostan-3-one. This steroid has been postulated as a possible intermediate in the metabolism of testosterone but up to now has been neither isolated from natural sources nor detected in metabolism experiments *in vivo* or *in vitro*.

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Clinical, Bacteriological, and Serological Observations of two Human Volunteers Following Ingestion of *Escherichia coli* O127:B8. (22338)

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An epidemic of infantile diarrhea due to *Escherichia coli* O127:B8 reported by Stulberg and co-workers(1) has been further studied serologically by Stulberg, Zuelzer and Page(2). In an effort to produce specific antibodies in humans to be used as positive control sera for the serological portion of their studies, 2 adult volunteers ingested O127:B8 organisms following the methods employed by Ferguson and June(3) and June, Ferguson and Worfel(4) in their classical feeding experiments using *E. coli* O111:B4 and O55:B5. Since volunteer studies using O127:B8 have not been published, and because clinical, bacteriological and serological results of seeming interest were obtained, this experience is being reported.

Methods. Two adults, white males, age 28 and 32, participated in this study. Each man was in general good health and had been free of diarrhea of any nature for at least one year prior to this experiment. *Inocula*: *E. coli* O127:B8 cultures obtained from 3 infants during their diarrheal disease(1) were inoculated separately on brain heart infusion agar slants. After 18 hours incubation at 37°C, the growth from each slant was harvested in saline. The 3 suspensions were pooled and washed once in saline. The pooled suspension was diluted in saline and colony counts estimated the inocula for the two volunteers to contain 4,000,000 and 8,000,000 organisms respectively. In each instance the inoculum was contained in 1 ml. Each inoculum was added to one-half pint of pasteurized milk which was kept on ice until ingested, approximately one hour later. A challenge inoculum of 8,000,000 organisms prepared in a similar manner was ingested by volunteer A 32 days following the initial ingestion. One pre-ingestion blood sample and 3 pre-ingestion rectal swabs were obtained from each volunteer. Following ingestion, rectal swabs and venous blood samples were collected daily for about 2 weeks and later at approximately 3 day intervals. A detailed record of symptoms was kept by each volunteer. *Bacteriological and serological examinations*: Rectal swabs were inoculated immediately on blood agar and MacConkey's bile salts agar plates. After 20 to 24 hours, colonies appearing on the blood plates were tested for agglutinability with OB rabbit antiserum prepared with *E. coli* O127:B8, strain 9782 obtained from Dr. William Ferguson. Standard bacterial agglutination tests using OB and O antigens prepared with *E. coli* O127:B8 culture No. 2220 were performed according to the method described by Kauffmann(5). Saline suspensions of the organisms grown for 18 hours on brain heart infusion agar were used as antigens. Hemagglutination tests were performed by a technic developed by Stulberg, *et al.*(2), based on the methods of Neter, *et al.*(6), and Wheeler and Taylor(7).

Results. Symptoms: Both volunteers experienced a sharply defined illness beginning with an explosive liquid stool. Liquid diarrhea, abdominal cramps, nausea and mild to moderate malaise were the predominating symptoms. In volunteer A the incubation period was 17 hours, the illness lasted 32 hours and with 4 diarrheal stools and an afebrile course was classified as mild. In volunteer B the incubation period was 7 hours, the duration 46 hours and with 10 diarrheal stools and a per oral temperature elevation of 100°F, the illness was classified as of moderate severity. No symptoms were recorded following the challenge ingestion by volunteer A.

The presence of *E. coli* O127:B8 in fecal cultures was determined by slide agglutination tests with 5 to 15 colonies from each blood plate. Organisms of this serotype were recovered from both volunteers 24 hours after ingestion and for a period of 5 days in one volunteer and 12 days in the other. In volunteer A, *E. coli* O127:B8 was found in the stools in increasing amounts and on the sixth day after ingestion, was the only organism present. The stool flora then gradually reverted to its pre-ingestion status. From the 13th through the 26th day 5 negative cultures were obtained. On the 32nd day a pre-challenge ingestion culture produced scanty growth of O127:B8. One day following the challenge ingestion moderate growth was present. Additional post-challenge cultures were not obtained.

Serological examination: Results of the standard agglutination and hemagglutination tests are shown in Table I. Serum hemagglutinins were detected as early as 3 days after ingestion and bacterial agglutinins with boiled cultures within 5 days. Titers with both tests continued to rise until the eighth or ninth day and remained near the peak level for the remainder of the initial experiment. Bacterial agglutinations with living cultures were, in every instance, negative at a serum dilution of 1:10. In volunteer A on the 32nd post-ingestion day bacterial agglutination was negative at a serum dilution of 1:10 while the hemagglutinin titer had dropped to 1:40. Three days following the challenge ingestion bacterial agglutinin and hemagglutinin titers were 1:40 and 1:160 respectively.

TABLE I. Results of Serological Examinations.

Days following ingestion	Agglutination titers* at different intervals					
	Volunteer A			Volunteer B		
	Bacterial O	Bacterial OB	Hemagglutination	Bacterial O	Bacterial OB	Hemagglutination
0	0†	0	20	0	0	10
1	0	0	20	0	0	10
2	0	0	40	0	0	20
3	0	0	80	0	0	40
4	0	0	80	0	0	40
5	10	0	80	10	0	40
6	20	0	320	20	0	80
7	160	0	320	40	0	80
8	80	0	320	40	0	160
9	160	0	640	40	0	160
12	80	0	320	40	0	80
15	80	0	320	20	0	80
18	80	0	320	20	0	80
32	0	0	40	nt		nt
3 (post-challenge)	40	0	160	nt		nt

* Titers are shown as reciprocal of highest dilution in which definite agglutination was observed.

† Zero indicates negative result at dilution of 1:10.

nt = not tested.

Discussion. Our results are interpreted as evidence of a definite bacterial, clinical and serological response by 2 adult human volunteers to the ingestion of *E. coli* O127:B8 organisms. The clinical responses, including the incubation periods and the duration of symptoms, are similar to those observed by Ferguson with serotypes O111:B4 and O55:B5. It is interesting to note, however, that volunteer B suffered an illness of moderate severity after the ingestion of only 4,000,000 organisms. In no instance did Ferguson record an illness of more than slight severity with dosages of less than 530,500,000 organisms with O111:B4 or 143,000,000 organisms with O55:B5. The question of possibly greater pathogenicity for adults of serotype O127:B8 in comparison with O111:B4 and O55:B5 is raised by these observations.

Serological tests showed comparable rises in antibody titer for the 2 volunteers, the hemagglutinin titers being consistently higher than the bacterial agglutinin titers. This is in agreement with the results obtained by Neter(8) in a comparison of the two methods, using sera obtained from volunteers in the feeding studies of Ferguson. The antibody response detected by bacterial agglutination when using boiled cultures as antigen could not be demonstrated when living cultures were used. This is in accord with Ferguson's results.

In both volunteers *E. coli* O127:B8 appeared in the stool 24 hours after ingestion of the cultures and increased in numbers during the following few days. The persistence of the organisms in substantial numbers for several days after the complete subsidence of symptoms is interesting and is in accord with the findings of Ferguson and co-workers with the O111:B4 and O55:B5 serotypes.

The results of the challenge feeding suggest that protection from clinical symptoms may have been afforded by the initial ingestion. It is interesting that the pre-challenge culture was positive although 5 previous cultures had been negative. Properly designed human experiments seem indicated to study a possible post-ingestion immune response and to delineate the duration and consistency of fecal elimination of the organism during convalescence.

Summary. 1. Two healthy, adult white, male volunteers ingested, respectively, 4 and 8 million organisms of *E. coli* serotype O127:B8. 2. Symptoms of mild to moderate gastroenteritis appeared in both subjects 7 to 17 hours after ingestion of the organisms and persisted for 32 to 46 hours. 3. By daily rectal swab cultures, *E. coli* O127:B8 organisms were recovered within 24 hours after ingestion and were found during 5 days in one and 12 days in the second volunteer. 4. Hemagglutinins were detected 3 days and bacterial agglutinins 5 days following ingestion. Antibody titers in both subjects rose rapidly, reaching peaks by the eighth to ninth day. Hemagglutination titers were consistently higher than bacterial agglutination titers. 5. Challenge ingestion of 8,000,000 organisms by one volunteer was followed by bacterial and serological response without appearance of clinical symptoms.

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Relation of Glucagon to A Cells of the Pancreas.*† (22339)

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In spite of the amount of work done on origin of glucagon, there is no agreement as to the site of its production(1-5). Some investigators found a significant reduction of glucagon in the pancreas of cobalt-treated guinea pigs and dogs(8,9); others found no diminution in glucagon content of cobalt-treated dogs(6,7); while others stated that the glucagon content of cobalt-treated rabbits was twice that of normal(4). However, these workers(4) found no glucagon in the pancreas of synthalin A treated rabbits. Some but not all controversies on origin of glucagon could be explained by differences in toxic effect on the A cells of different animals(10,11). It has recently been demonstrated by one of us that the uncinate process of the dog pancreas is normally devoid of A cells(12) and that no significant hyperglycemic effect can be obtained with extracts from such an area while there is a marked hyperglycemic effect with extracts from the A cell containing portion of the pancreas(13). Because of the specific morphological differences in the dog pancreas, it was thought that other differences rather than the presence or absence of A cells might explain the regional differences in the glucagon content of the dog pancreas. This hypothesis had to be seriously entertained in view of the conflicting reports on the relation of glucagon to the A cell(1-5). To clarify this relationship, an attempt was made to destroy the A cells of the dog pancreas with cobalt but unfortunately we were unable to do so, although they were easily damaged in the guinea pig(11). Experiments were carried out then with the object of producing complete disappearance of the A cells of the guinea pig with the hope that a pancreas with these changes would be analogous with respect to its morphology and glucagon content to that of the uncinate process of the dog.

Material and methods. A total of 64 guinea pigs of both sexes were divided as follows: *Group 1:* Five guinea pigs weighing between 600 and 1200 g were used for the study of possible regional differences in the distribution of islet cell types in the guinea pig. The entire pancreas of these animals was embedded and sectioned and in none of the representative sections could we find evidence of any regional differences. *Group 2:* The pancreases of 4 untreated guinea pigs weighing between 900 and 1200 g were pooled and used for glucagon extraction. A small portion of each gland was first taken for microscopic examination. *Group 3:* Four guinea pigs weighing between 400 and 700 g and 6 guinea pigs weighing between 1100 and 1400 g received daily subcutaneous injections of 20 mg of cobalt chloride (B.D.H. Analar) per kg of body weight as a 1% solution for as long as 7 days. The animals of the younger group were all killed at the seventh day. Of the older group, 5 died between the third and fourth days; the survivor was killed on the fifth day. The entire pancreas of all these animals was embedded and studied as in Group 1. The older animals showed the most extensive A cell lesions, but were also more susceptible to the lethal effect of cobalt. The extent of damage to the A cells was variable from one animal to another but of similar degree throughout the pancreas of any one guinea pig. On this basis in subsequent experiments the extent of A cell damage of the portion of pancreas used for glucagon extraction was assumed to be identical to the small one used for histological examination. *Group 4:* Because most animals lost about 10% of their weight during the cobalt treatment 9 guinea pigs were partially starved to make them lose 10% of their weight within 5 days so as to exclude weight

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† Presented at Meeting of Canadian Physiol. Soc., London, Ontario, Oct. 14, 1955.

loss as the cause of the A cell lesion in the cobalt treated guinea pigs. None of these starved animals showed A cell damage. *Group 5:* Thirty-six guinea pigs weighing between 900 and 1200 g were treated with cobalt chloride as in Group 3; fourteen died and were discarded, the remaining 22 were killed between the fifth and seventh day after the beginning of the treatment. A small portion of the pancreas was obtained from each of these animals for microscopic study, the remainder of the organ was used for glucagon extraction. Histologically, the extent of the A cell lesion was graded for each pancreas from 1+ to 4+. Eight animals had 1+; 5 had 2+; one had 3+; and 8 had 4+ lesions of their A cells. Extracts from animals having the same degree of damage were pooled in groups up to 4. All animals had free access to water and Purina rabbit chow supplemented with fresh lettuce and carrots. All guinea pigs were killed at 6 hours after the last injection of cobalt by exsanguination under ether anesthesia. Glucagon was extracted by the method of Best, *et al.*(14), each pancreas was extracted individually and then pooled as described above. The pooled extracts were dried by evaporation in the cold. Every pooled extract was tested in triplicate following the procedure suggested by Staub and Behrens(15). The presence of glucagon was demonstrated by its hyperglycemic effect on fasted, anesthetized cats. The dry extract in an amount equivalent to 2 g of fresh pancreas was dissolved in 2 ml of normal saline (pH 2.5) immediately before injection. The solution was injected intravenously and blood samples were taken prior to and 5, 10, 15, 20 and 25 minutes after the injection. Individual blood sugar determinations were done in duplicate with a modification of the Folin-Wu method. The extract from the animal with a 3+ lesion was tested only once because of insufficient material for more determinations. Tissues for histological examination were fixed in Zenker-Formol, and were processed and stained following the methods previously described(16). Paraffin sections were cut 2.5 mu thick and stained with Gomori's chrome alum hematoxylin stain, the aldehyde fuchsin stain of Gomori(17) and a recent modification of Masson's trichrome(11).

Results. Morphology. The A cell lesion of the cobalt-treated guinea pig was qualitatively identical in all animals. Cobalt produces a progressive and complete degranulation of the A cell, while its cytoplasm and nucleus remain unaffected. With further treatment, the degranulated A cell undergoes vacuolization of its cytoplasm (Fig. 1,2). It was not possible, however, to see any necrotic cells in the pancreases of cobalt-treated guinea pigs. Our grading of the A cell lesions was based on the proportion of A cells showing complete degranulation or more advanced changes. We considered a 1+ lesion to be present in all those cases in which some cells were observed. When about half of the A cells were so damaged, it was graded as a 2+ lesion. When only a few granulated A cells could be found, the lesion was classified 4+. An attempt was made to interpolate a 3+ lesion, but this could be done on only one occasion.

Glucagon content of the pancreas of untreated guinea pigs: The extracts from the pancreases of these animals invariably showed when tested a marked hyperglycemic effect on the fasted anesthetized cats. Their blood sugar rose from 50 to 153 mg% above the initial level within 15 minutes after the injection of the extracts as indicated in Fig. 3.

Glucagon content of cobalt-treated guinea pigs: When tested, pancreatic extracts from animals having 1+ and 2+ lesions showed a response similar to that of the untreated animals. As noted in Fig. 3, the extracts from a 2+ lesion gave a rise of blood sugar varying from 73 to 104 mg %. In contrast with these results the extract from animals having a 4+ lesion showed on only one occasion a rise of 4 mg % above the initial level. This was followed by a decrease in the blood sugar from 40 to 62 mg % within 25 minutes. The pancreatic extract from the animal having a 3+ lesion showed a rise of blood sugar of 16 mg % above the initial level.

Discussion. An important finding in the present work is the lack of a hyperglycemic effect of the pancreatic extract from animals having 4+ lesions, particularly since the hy-

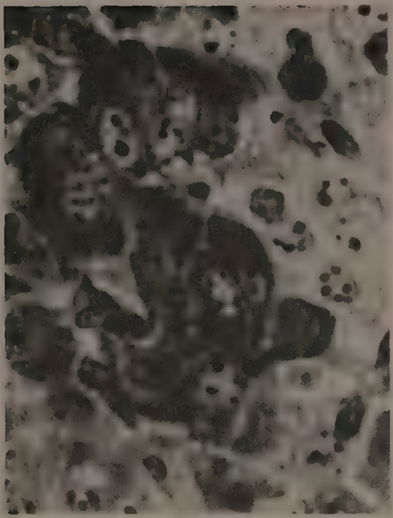

FIG. 1. Pancreatic islet from normal guinea pig showing well granulated A cells in black. Masson's trichrome × 900.

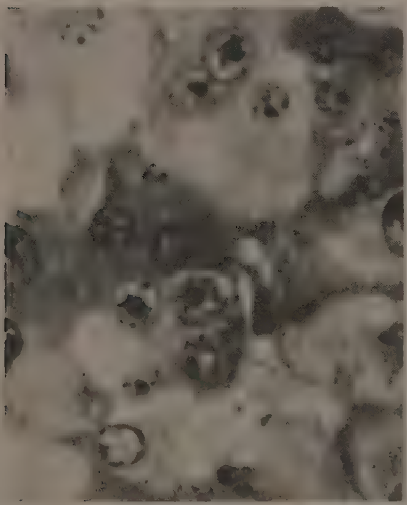

FIG. 2. Pancreatic islet from a cobalt-treated guinea pig. A cells are severely degranulated or vacuolated. They appear clear in the picture. Masson's trichrome × 900.

perglycemic effect of the extract from animals having a 1+ and 2+ lesion is indistinguishable from that of the untreated ones. Our results suggest that the pancreatic extracts from cobalt-treated guinea pigs fail to elicit a hyperglycemic effect, as is the case with extracts from the A cell-free portion of the dog pancreas when the number of granulated A cells present is reduced to a certain minimum which apparently is achieved only in our 4+ lesion. Because 4+ lesions occurred in only 40% of cobalt-treated animals, it is possible that the 60% reduction of glucagon content obtained by Vuylsteke *et al.*(8) in their pooled extracts from pancreases of cobalt-treated guinea pigs was due to the fact that these authors did not grade the extent of their lesion. From our experience with cobalt in various species(11) from the work with the uncinate process of the dog(12,13) and since none of the substances toxic for the A cell have resulted in pancreas completely devoid of them(1,3,4), it seems that the conflicting results on the relation of glucagon to the A cell may be due to the great hyperglycemic activity of minute amounts of glucagon remaining in the pancreas when a few granulated A cells are present. We are, however, unable to explain on the basis of our results the work of Fodden and Read(4) who found that extracts from cobalt-treated rabbits had double the normal amount of glucagon while that of synthalin-treated ones had none despite the fact that A cells were severely damaged in both groups. It is unfortunate that these investigators did not make a detailed comparison of the A cell lesion as it occurred in

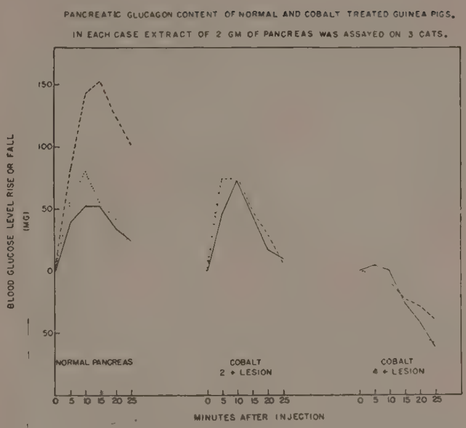

FIG. 3. Typical blood sugar curves in fasting and anesthetized cats after intravenous injection of pancreatic extracts from normal and cobalt-treated guinea pigs.

the rabbits treated with these two substances. The results of our work are interpreted as strongly suggestive that glucagon originates from the A cells. Quantitative determination of the glucagon content of pancreas treated with drugs toxic for the A cell together with detailed morphological study of the type of A cell lesion, will aid in the elucidation of this problem.

Summary. Pancreatic extracts of cobalt-treated guinea pigs with a 4+ lesion fail to produce a significant hyperglycemia when assayed in cats; however, if well-granulated A cells remained in amounts roughly estimated above 25% of the normal, the extracts from these pancreases invariably elicited a hyperglycemia indistinguishable from that of the normal. These results are interpreted as strongly suggestive that glucagon originates from the A cells.

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Effect of Splenectomy on Antibody Formation in Cortisone Treated Rats. (22340)

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The hemolysin formation in rats after a single intraperitoneal injection of sheep erythrocytes was the object of a previous study (1). It was suggested that at a large dose of the antigen (about 2 ml of a 10% suspension) a considerable part of the sheep cells enters the blood stream and is taken up by the spleen. The spleen may then be responsible for a large fraction of the circulating antibodies measured. However, at an antigen dose 30-40 times smaller, splenectomy preceding(2,3) or subsequent to (unpublished experiment) the intraperitoneal antigen injection did not appreciably alter the hemolysin response. In another series of experiments some factors which condition the antibody-reducing effect of cortisone were studied(4). It was shown that the hemolysin response after a large intraperitoneal dose of sheep cells was less affected by cortisone than that after a small one. One possible explanation involved the assumptions: 1) that the spleen produces a dominating part of the hemolysins with a large antigen dose but not with a small one, and 2) that splenic antibody production is more resistant to cortisone than is extrasplenic antibody formation. The effect of cortisone should then be less with a large than with a low antigen dose.

The following experiment was an attempt to verify the two assumptions.

Material and methods. Animals. White

TABLE I. Effect of 4 mg Dose of Cortisone on Hemolysin Response in Sham Operated and Splenectomized Rats. Animals were immunized by one intraperitoneal injection of sheep erythrocytes on day 0.

Group	Operation	Treatment	Initial avg wt, g	Hemolysin titer, mean ± stand. error of mean†		
				Day +4	Day +5	Day +8
I	Sham	C* day −8 to +1	208	3.26 ± .52 (9)	6.81 ± .38 (9)	6.77 ± .55 (9)
II	”	No	204	5.19 ± .45 (8)	7.50 ± .29 (8)	6.41 ± .36 (8)
III	Splenectomy	C day −8 to +1	200	.00 (10)	.57 ± .37 (10)	1.40 ± .62 (9)
IV	”	C day −2 to +1	196	.07 ± .03 (9)	2.77 ± .47 (9)	3.94 ± .49 (9)
V	”	No	214	1.87 ± .49 (7)	5.57 ± .43 (7)	5.37 ± .48 (6)

* C = Cortisone. † Figures within parentheses are No. of animals.

male rats weighing 170-200 g were allotted at random to the experimental groups(1). Blood samples of 1 ml were taken from tails. *Splenectomy and sham operation.* All operations were performed under evipal sodium anesthesia on the 1st day after the antigen injection. After midline abdominal incision the spleen was exteriorized. In the sham operation, the spleen was replaced in the abdomen. When splenectomy was performed the spleen was removed after ligation of the pedicle. *Hormone.* Cortisone acetate* was given as daily intramuscular injections in a dose of 4 mg per 100 g body weight (designated "the 4 mg dose"). *Antigen.* Fresh sheep erythrocytes were washed 3 times with saline and diluted with saline to a 10% suspension. A small quantity of India ink (dilution in antigen 1:2000) was added. The rats were immunized by a single intraperitoneal injection of 1 ml of the antigen suspension per 100 grams initial body weight (Table I). At the end of the experiment all animals were autopsied to determine whether antigen had been deposited in the peritoneal cavity (traces of carbon). The day of antigen injection is called day 0, days before and days after are conventionally indicated by minus and plus respectively. *Titration of hemolysin.* Inactivated sera were titrated in 2-fold serial dilutions. Hemolysins were determined by a standard procedure(1) using 4 units of complement, incubation at 37°C for 30 minutes, and subsequent centrifugation. The degree of hemolysis was estimated by the aid of a hemolytic scale. The 50% end point was calculated by interpolation. The titers are expressed as the negative ^{2}logarithms of the serum dilution, taking dilution 1:6.25 as a provisional zero point (1:12.5 = 1; 1:25 = 2, etc.). All sera from one bleeding were titrated simultaneously.

Experimental. A scheme of the cortisone treatments and the operations is given in Table I. Two groups of 12 rats were given the 4 mg dose of cortisone for 10 days beginning 8 days before the antigen injection (I and III). One group of 9 rats received the same dose of cortisone for 4 days beginning 2 days before the antigen injection (IV). Another 2 groups of 9 rats had no treatment (II and V). One day after antigen injection, splenectomy was performed on the following groups: Group III, treated with cortisone for 10 days; Group IV, treated for 4 days, and Group V, non-treated. The remaining 2 groups (I and II) were sham operated. Five animals died during or soon after the operation. Another 5 animals died later, probably due to infections. In 3 of these the intraperitoneal deposition of the antigen could not be verified. The data of these rats have been excluded. The animals were bled on the 4th, 5th and 8th day after the antigen injection.

Results. Splenectomy produced a significant reduction of the hemolysin level on day +4 (Fig. 1 and Table I). This effect appeared to be less pronounced on day +5 and was no longer significant on day +8.

In sham operated animals the 10-day cortisone treatment produced a probably significant ($P<0.02$) titer reduction on day +4 (Table II). At the later bleedings this effect was no longer manifest. In splenectomized rats the same treatment had a significant effect on all bleeding days. On day +5 and +8 the 10-day cortisone treatment seemed to be much more effective in splenectomized

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* Cortone, Merck and Co., furnished through kindness of Erik Lindblom and Co., Stockholm.

TABLE II. Mean Titer Difference between Non-Treated Groups (II and V) and the Respective Similarly Operated Cortisone-Treated Groups (See Table I).

Operation	Cortisone, No. of days	Titer reduction, mean ± S.E. of mean Day +4	 Day +5	 Day +8
Sham	10	1.93 ± .69*	.69 ± .49	–.36 ± .67
Splenectomy	10	≥1.87 †§	5.00 ± .57‡	3.97 ± .86‡
"	4	1.80 *§	2.80 ± .66‡	1.43 ± .72

* Significance level 5%. † Significance level 1%. ‡ Significance level .1%.

§ This significance was obtained by comparison of intra-group distributions of values above and below the general mean of the two groups, see Mainland(7).

animals than in those sham operated. To test the significance of this difference, an analysis of variance(5) was used with two bases of classification: splenectomy and cortisone treatment (10 days or none). The interaction term proved the difference significant at the 0.001 level.

In splenectomized animals cortisone treatment for 4 days produced a significant reduction of the hemolysin level on day +5 but not on day +8 (Table II).

Discussion. Splenectomy produced a significant reduction of the hemolysin level only on the 2 first bleeding days (Fig. 1). Thus the splenectomized animals seemed to have a slower rise of antibody but may actually have formed as much antibody as the controls. Taliaferro and Taliaferro(6) have reported similar observations in rabbit experiments where the antigen was given intravenously. These authors suggest "that the spleen in intact animals usually forms a large part of the antibody which accumulates to give the initial peak serum titer, and then abruptly ceases to form antibody." Such an early transient role of splenic hemolysin formation may also explain the present findings.

Administration of the 4 mg dose of cortisone for 10 days, begun 8 days before the antigen injection, was significantly more effective in splenectomized than in sham operated animals on day +5 and +8. In fact cortisone treatment for 4 days, started 2 days before the antigen injection, was enough to produce a significant reduction of the antibody level on day +5. In non-splenectomized animals this short period of hormone administration has failed to significantly affect the hemolysin response to this antigen dose (about 2 ml of a 10% suspension) but has repeatedly suppressed the antibody level on day +5 at a 30 times smaller sheep cell dose (4).

The present results indicate that splenectomy has lowered the resistance to cortisone of the hemolysin formation after a large intraperitoneal antigen dose. The mechanism of this effect is not clear. The result of splenectomy in animals not treated with cortisone (c.p. day +8, Fig. 1) does not lend support to the hypothesis that most of the hemolysins, produced after a large antigen dose, emanate from the spleen. Consequently the present data do not indicate whether or not the splenic antibody formation is more resistant to cortisone than the extra-splenic. The experiment has not clarified the cause of the

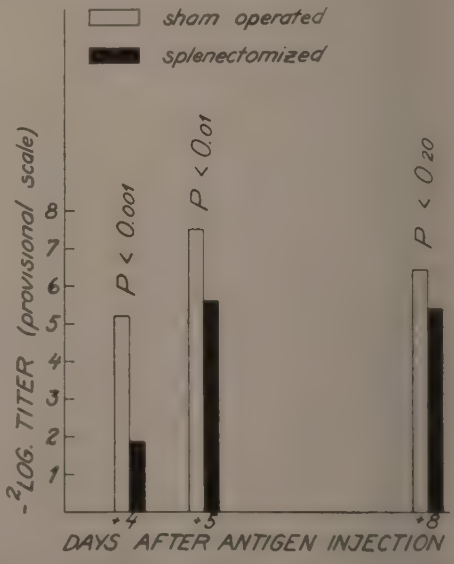

FIG. 1. Effect of splenectomy on average hemolysin response after a single intraperitoneal inj. of sheep erythrocytes. P values indicate level of significance of mean difference.

increased resistance of hemolysin production after a large intraperitoneal sheep cell dose.

Summary. 1. Splenectomy, performed one day after an intraperitoneal injection of a large dose of sheep erythrocytes, reduced the initial part of the hemolysin response, but did not significantly affect a later part. 2. The antibody-reducing effect of cortisone was significantly greater in splenectomized than in sham operated rats.

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Simplified Method for Calculating Flow, Mean Circulation Time and Downslope from Indicator-Dilution Curves. (22341)

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The use of indicator-dilution curves to measure cardiac output and "central blood volume" is widespread and of proved value (1-4). When the curves are markedly prolonged, as in heart failure, the calculations as ordinarily performed are time consuming because of the necessary integrations of the curves. Two methods of integration of the curves are in general use. The first consists of arithmetic summation of all concentrations each second from appearance time to some time later—usually through one or two cycles of semi-logarithmic paper. The second method involves replotting the curves (after extrapolation on semi-logarithmic paper) on linear paper and integrating planimetrically. The calculation of mean circulation time is done by finding the center of gravity of the linearly plotted curve on cardboard(2), or else by summing arithmetically all the products of time and concentration from the semi-logarithmic plots and dividing by the arithmetic or planimetric integration. Lewis(5), described an ingenious method of calculating the cardiac output from indicator-dilution data which eliminated the necessity for plotting the curves. Though generally useful, this method does not permit calculation of mean circulation time. In addition errors in samples which might become apparent from plots of the data could be missed more easily.

This paper proposes a method for accurately obtaining the integral, slope and mean circulation time of an indicator-dilution curve in a short time, with relatively simple calculations. The method involves a combination of arithmetic integration (summing of concentration) of a small portion of the curve, and mathematical integration of the remainder.

Method. Semilogarithmically plotted indicator-dilution curves generally can be divided into two portions. The first is the rapidly rising upslope and the curved portion of the downslope; the second is the linear portion of the downslope, extrapolated toward infinite time. It becomes apparent that the integral of the total curve is the sum of 2 areas—the area from appearance time to beginning of linearity of the downslope, and the area from the beginning of the downslope to infinite time (Fig. 1). The area of the first portion can easily be obtained by summation. Because of the steep rise and relative sharp-

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[*] Helen H. Millenson fellow in Cardiology Research.

[†] Fellow, Life Insurance Medical Research Fund.

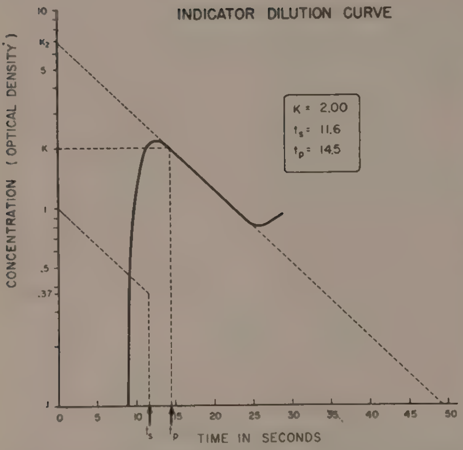

FIG. 1. Sample indicator dilution curve analyzed according to method described. (See text for symbols.)

ness of the peak only a few concentrations have to be summed—the integration of the linear portion can be determined graphically as follows, and can be added to the first integral to obtain the total area of the curve: 1. A line perpendicular to the time axis is drawn through the highest concentration on the linear downslope. The intercept of this line with the baseline is noted and recorded as t_p. 2. A line parallel to the downslope is then constructed passing through the value 1.0 of the concentration axis. Another perpendicular to the time axis is dropped from the intersection of this line with the 0.37 concentration line. The intercept of this perpendicular with the time axis is then noted and recorded as t_s. This value is the reciprocal of the downslope S and when multiplied by the flow would give the "lung" volume of Newman(4). 3. A fourth line is now drawn parallel to the time axis through the beginning of the linear downslope. The point at which this line crosses the concentration axis is noted and recorded as K. 4. The product Kt_s is equal to the integral of the straight line portion of the curve. As stated previously this integral is added to the arithmetically obtained integral of the first portion of the curve to obtain the total area. The amount of indicator injected into the circulation is divided by the total integral to obtain flow.

To calculate the mean circulation time of the entire curve we proceed as follows: 1. The product Kt_s $(t_s + t_p)$ is obtained. 2. The concentration for each second, from appearance of indicator to beginning of linear downslope, is obtained from the curve and multiplied by its respective time. These products are summed. 3. This sum is added to the value obtained from step 1. above. 4. The final sum (from 3. above) is divided by the previously obtained total area of the curve. The result is the mean circulation time, T_m. The validity of the method is apparent from examination of the mathematical derivation in the appendix.

Summary. 1. A new graphical method for calculating flow, downslope and mean circulation time from semilogarithmic plots of indicator-dilution concentration-time curves is described. 2. The advantages of employing this method of calculation include speed and accuracy.

APPENDIX: *Mathematical Proof of Method.*

I. Slope determination of indicator dilution curves. The slope is usually obtained from formula

$$(1)\quad \text{Slope } S = \frac{\log_e C_2 - \log_e C_1}{T_2 - T_1}$$

This formula is applicable with any values of C_2 and C_1 obtained from the downslope. Since the slope of any line parallel to the downslope is the same as the downslope we can construct a line parallel to the downslope passing through the 1.0 concentration value. Slope of this line would be identical with slope of our original. We can choose any 2 values of C_2 and C_1 to determine the slope. Choosing values $C_1 = 1.0$ and $C_2 = 0.37 = e^{-1}$, the corresponding times for these concentration values would be T_1 and T_2. Since our line intersects the concentration axis at 1.0 at time 0 then $T_1 = 0$. Substituting into our formula (1) we obtain

$$(2)\quad \text{Slope } S = \frac{\log_e C_2 - \log_e 1.0}{T_2 - 0}$$

Since $\log_e 1.0 = 0$ then

$$(3)\quad S = \frac{\log_e C_2}{T_2}$$

Since we have set $C_2 = e^{-1}$ we now substitute this and get

$$(4)\quad S=\frac{\log_e e^{-1}}{T_2}$$

Since $\log_e e^{-1} = -1$ then

$$(5)\quad S=\frac{-1}{T_2}\quad \text{or}$$

$(6)\ T_2=\frac{-1}{S}$, the minus sign indicating the direction of the slope.

Thus time T_2 (the time corresponding to a C_2 of 0.37) is equal to reciprocal of the downslope.

II. Flow determination

Total area of the curve can be represented by the integral $\int_0^\infty Cdt$. By drawing a line perpendicular to the time axis through highest portion of the linear downslope, curve can be divided into 2 portions whose total area is the sum of the area up to the perpendicular line and the area from the perpendicular to infinite time. Expressed mathematically:

$$\int_0^\infty Cdt = \int_0^{t_p} Cdt + \int_{t_p}^\infty Cdt$$

Value of the integral $\int_0^{t_p} Cdt$ is obtained arithmetically by summation.

The value of the integral $\int_{t_p}^\infty Cdt$ can be shown to be K/S as follows:

If a line parallel to the downslope is drawn through the concentration axis at K, (highest value of linear downslope) then the area under this line must be equal to area under downslope since both triangular areas (on semilogarithmic paper) are congruent and at the same concentration level. The equation representing this constructed line is

$$(7)\quad C = Ke^{-St}$$

its area is

$$(8)\quad \text{Area} = \int_0^\infty Ke^{-St}dt = \text{area under downslope} = \int_{t_p}^\infty Cdt$$

Since K is constant we can write

$$(9)\quad \int_{t_p}^\infty Cdt = K\int_0^\infty e^{-St}dt$$

Integrating the right hand expression we get

$$(10)\quad \int_0^\infty Cdt = K\left[\frac{-1}{S}e^{-St}\right]_0^\infty = K/S$$

By adding this area K/S to the arithmetically obtained value of $\int_0^{t_p} Cdt$, we obtain the value of the total integral to infinite time. By dividing the amount of indicator injected, Q, by this integral the flow results from the formula

$$(11)\quad \text{Flow} = \frac{Q}{\int_0^\infty Cdt}$$

III. Mean circulation time determination.

The mean circulation time is defined as

$$(12)\quad T_m = \frac{\int_0^\infty Ctdt}{\int_0^\infty Cdt}$$

Value of denominator is determined as described above. Value of numerator is derived as follows:

The integral $\int_0^\infty Ctdt$ can be divided into 2 parts (by a line drawn through the highest point on the downslope perpendicular to the time axis) so that

$$(13)\quad \int_0^\infty Ctdt = \int_0^{t_p} Ctdt + \int_{t_p}^\infty Ctdt$$

Value of $\int_0^{t_p} Ctdt$ is obtained arithmetically by summing all the products of concentrations and time each second up to t_p. To this sum is added value of integral $\int_{t_p}^{\infty} Ctdt$ determined as follows:

Since the curve of concentrations against time plotted semilogarithmically is a straight line from t_p to infinity this curve can be expressed by the formula

$$(14)\quad C = K_2\, e^{-St}$$

where K_2 is the intercept of this line extrapolated back to the concentration axis (zero time).

Substituting (14) into the integral we are trying to determine we can say

$$(15)\quad \int_{t_p}^{\infty} Ctdt = \int_{t_p}^{\infty} tK_2e^{-St}dt$$

Since K_2 is a constant we can write

$$(16)\quad \int_{t_p}^{\infty} Ctdt = K_2 \int_{t_p}^{\infty} te^{-St}dt$$

Solving we get

$$(17)\quad \int_{t_p}^{\infty} Ctdt = K_2\left[\frac{e^{-St}}{(-S)^2}(-St-1)\right]_{t_p}^{\infty}$$

$$= K_2e^{-St_p}(St_p + 1)$$

The expression can be simplified as follows:

The area under the downslope from t_p to infinity is equal to the area under the curve obtained by drawing a parallel line through the concentration axis (zero time) at K. Equating these areas we get

$$(18)\quad \int_{t_p}^{\infty} K_2e^{-St} = \int_{0}^{\infty} Ke^{-St}$$

and solving

$$(19)\quad K_2\, e^{-St_p} = \frac{K}{S}$$

or

$$(20)\qquad K_2 = \frac{K}{e^{-St_p}}$$

substituting the value for K_2 from (20) into equation (17) we get

$$(21)\quad \int_{t_p}^{\infty} Ctdt = \frac{K}{S^2}(1 + St_p) = \frac{K}{S}\frac{1}{S}(1 + St_p)$$

$$\text{Since } \frac{1}{S} = t_s$$

$$(22)\quad \int_{t_s}^{\infty} Ctdt = Kt_s\,(t_s + t_p)$$

Adding this to the arithmetically obtained value for $\int_{0}^{t_p} Ctdt$ we get the entire value for $\int_{0}^{\infty} Ctdt$ which when substituted into equation (12) gives mean circulation time.

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Demonstration of a Toxic Factor(s) in Thermal Death.* (22342)

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Although fluid balance may play an important role in the problem of shock and thermal death, other investigations have supported the theory that toxic substances are released into the circulatory system after scalding and that this toxic material is responsible for death of the organism(1-3). Helderweirt *et al.*(4), experimenting with heat death in rabbits, noted that even when lowered blood volume of scalded rabbits is experimentally returned to normal, the organism succumbs to "another trouble still unknown." The work of LaFontaine(5) and Simonart(6,7) indicates the importance of a toxic factor in blood of scalded frogs. Although the precise function of toxic substances is still argued, it has been suggested that the toxic factor is in some way connected with protoplasmic and blood clotting(8-10).

The arguments for and against the toxic factor theory have, however, been based on experiments involving only vertebrate organisms. It is, therefore, the purpose of this study to determine if a toxic substance could be found in marine invertebrates. Although preliminary experiments uncovered several invertebrate organisms which appeared to release a toxic substance when scalded (for example, starfish), this paper will deal solely with an investigation on the marine worm, *Phascolosoma gouldii*. One can easily obtain fairly large quantities of coelomic fluid from this thick-walled, unsegmented annelid. Another advantage in using this animal is that it does not undergo autotamy when subjected to scalding, a problem frequently encountered when experimenting with many other invertebrates.

Materials and methods. In the experiments to be described, both male and female worms *(Phascolosoma gouldii)* ranging from 3-5 inches in length were used. When scalding was necessary, the posterior half of the animal was immersed for 90 seconds in hot sea water (76 ± 1°C). Following this treatment the animals were immediately returned to finger bowls containing sea water and maintained at a relatively constant temperature by stacking the bowls on sea water tables. The water within the finger bowls was changed at least once daily. In those experiments involving dialysis, dialysis membrane having a flat diameter of 1¹⁄₁₆ inches was washed both inside and out, and the samples dialyzed against large volumes of running sea water (approximately 500 ml per minute) for 12 hours. The end point used in determining the survival time of worms is probably subject to an error of at least ± 2-3 hours. These organisms were considered dead when pinching them with a forceps no longer produced a response such as muscular contractions. Unless otherwise stated, in all experiments a minimum of 15 animals was used to determine average survival time. Eggs of the sea urchin, *Arbacia punctulata*, were obtained by electrical stimulation, as described by Harvey(11). The eggs were washed several times in sea water and fertilized by addition of diluted sperm. The per cent cleavage was determined by counting a minimum of 100 eggs per dish.

Results. Suggestive data for the release of a toxic substance was first obtained when comparing the survival time of scalded worms to that of worms ligated prior to scalding. Results indicated that, although scalded animals would die in about 21 hours, the survival time could be increased merely by securing a ligature (a heavy thread or Hoffman clamp) just anterior to the heated region, prior to scalding (Table I). It can be seen that by preventing the coelomic fluid in the scalded portion of the animal from mixing with the anterior portion, the survival time was increased. The fact that survival time could

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TABLE I. Effect of Pre-Scalding Ligature on Average Survival Time (in Hr ± S.D.) of Scalded Worms. Posterior half of organism immersed in sea water at 76°C for 90 sec.

Non-constricted animal		Animal constricted by ligature	
Scalded	Control	Scalded	Control
21 ± 13	Infinite	74 ± 29	120 ± 79

not be increased any longer than 3- or 4-fold may be explained on the basis of a leak in the ligature, but more likely by the fact that the procedure of ligating is in itself sufficient to cause death in 120 hours.

In an attempt to isolate any toxic material that was present in scalded worms, coelomic fluid was removed from worms 15 minutes after scalding by piercing the thick muscular wall with an 18 gauge hypodermic needle attached to a 5 ml syringe. The coelomic fluid was then centrifuged and 1 ml aliquots of the supernatant injected cephalad into the posterior end of normal recipient worms. As shown in Table II, coelomic fluid from scalded animals proved toxic when injected into normal recipients. The controls, when injected with 1 ml of cell-free coelomic fluid obtained from unheated *Phascolosoma,* lived indefinitely. It appears, then, that the act of scalding releases a toxic substance which is found in the coelomic fluid and is capable of causing death when injected into normal animals of the same species.

Origin of the toxic factor. Although the toxic substance is found in the coelomic fluid, the question arises as to whether the toxin is released from coelomic fluid itself or if it originates from another source. In an attempt to answer this question, experiments were carried out in which the coelomic fluid was removed from normal worms, the coelomic cavity flushed out (with sea water) and finally refilled with sea water. This "sea-water containing" worm was then scalded and the fluid within the coelomic cavity removed, centrifuged and 1 ml aliquots injected into normal recipient worms. The results obtained from this type of experiment showed that toxin was not released in those scalded animals where sea water was injected into the coelomic cavity in place of coelomic fluid. Such experiments suggest that the toxic substance originates in the coelomic fluid. However, the possibility still exists that, in order for the toxin to be released, an inter-action may be necessary between the coelomic fluid and the rest of the worm. In the following experiments the coelomic fluid from normal animals was removed, placed in test tubes and immersed in a water bath at 76°C for 90 seconds (2 minutes in instances where the diameter of the tubes was substantially larger than the diameter of worms). This heated coelomic fluid was cooled, centrifuged and 1 ml aliquots of the supernatant injected into normal worms. The results show that the toxic factor can be released merely by heating coelomic fluid *in vitro* since those animals receiving injections of this fluid succumb in about 40 hours. (Controls receiving 1 ml injections of unheated coelomic fluid lived indefinitely.) It appears, then that the toxin can be released without any interaction between coelomic fluid and the rest of the organism. Additional experiments were carried out in which pieces of body wall, as well as viscera, were heated *in vitro* in the presence of small volumes of sea water; but the supernatant, when injected into recipient worms, proved non-toxic.

TABLE II. Effect of Cell-Free Coelomic Fluid Taken from Scalded Worms on Average Survival Time (in Hr ± S.D.) of Normal Worms.

Worms inj. with coelomic fluid from scalded donor	Worms inj. with coelomic fluid from normal donor
29 ± 15	Infinite

By separating coelomic fluid into two portions, (1) the cells of the coelomic fluid—which were re-suspended in sea water and (2) the cell-free supernatant, it was shown, after heating at 76°C, that the toxic factor was released only in that fraction of coelomic fluid containing the cells. Thus the origin of the toxic substance released in this marine annelid after scalding appears to be in the cells of the coelomic fluid. It might be brought out at this point that preliminary experiments suggest that the toxin can be released by treatment other than heat—particularly homogenization.

Characteristics of toxic factor. The series of results, tabulated in Table III, show that

TABLE III. Effect of Heat and Dialysis on Toxic Factor Activity. Average survival time (in hr ± S.D.) of normal worms receiving 1 ml injections of precipitate-free coelomic fluid.

Cell-free coelomic fluid of scalded worm		Cell-free coelomic fluid of normal worm	
Autoclaved	Autoclaved & dialyzed	Autoclaved	Autoclaved & dialyzed
32 ± 14*	77 ± 32	Infinite*	Infinite

* Only 9 worms injected.

the toxic factor is still active after autoclaving (15 lb pressure per square inch). This heat-stable toxin appears to be a relatively large molecule (or at least tied up with a large molecule) in that after prolonged dialysis against running sea water, the toxic fraction still proves fatal when injected into normal recipients (Table III).

Experiments have shown that the toxic factor was not ether-soluble since ether extracts of toxic coelomic fluid, after having been evaporated and redissolved in sea water, had no apparent effect when injected into normal worms. The ether-insoluble portion still exhibited its toxicity. Although the toxic coelomic fluid has a lower pH than does the normal (normal = pH 7.8; toxic = 6.7), readjusting the hydrogen ion concentration, followed by injection of normal worms, shows that the toxicity was not due to any pH change. In like manner, it was shown that the toxicity was not due to any potassium differences that may be present between experimental and control coelomic fluids.

In an attempt to further isolate the toxic factor, samples of the coelomic fluid were heated *in vitro*, centrifuged and the supernatant autoclaved. The protein coagulated by autoclaving was then centrifuged off and the supernatant then saturated with ammonium sulfate. This resulted in the appearance of a flocculent precipitate. These fractions were separated by centrifugation and the precipitate redissolved in sea water (a volume equal to that of the supernatant). The 2 fractions, the supernatant and the precipitate, were then dialyzed until all traces of ammonium sulfate had been removed. When 1 ml aliquots of these fractions were injected into normal worms (Table IV) the fraction containing the precipitate, which was "salted out" by saturation with ammonium sulfate, proved toxic.

It might be noticed, when comparing the data presented in Tables II, III and IV, that animals injected with samples of coelomic fluid that have been dialyzed survive over twice as long as those animals injected with non-dialyzed coelomic fluid. These data suggest the possibility that two types of toxic molecules are released after scalding—one dialyzable and the other non-dialyzable.

Semi-micro assay. Since the only method of assay has been that of injecting fluid into recipient worms, a procedure requiring large volumes of toxin, it was felt that a micro assay was necessary, particularly if chromatographic studies were to be pursued. Eggs of the sea urchin, *Arbacia punctulata,* are relatively sensitive to the toxic factor, for when these eggs were fertilized in sea water and 5 minutes later immersed in the toxic fraction, cell division was substantially inhibited. It has been shown that only 6% of the eggs undergo cleavage when immersed in a 6.25% solution of cell-free coelomic fluid taken from scalded worms (diluted in sea water). Further proof that the factor inhibiting cell division is the same factor responsible for the toxic effects in the worm is seen in experiments in which fertilized *Arbacia* eggs were immersed in the fraction precipitated by ammonium sulfate (similar to those used in Table IV). As can be seen in Table V, cell division was completely inhibited in such instances although the various controls underwent substantial cleavage.

Summary. In a study of heat death, it has been shown that the marine annelid, *Phascolosoma gouldii,* releases a heat stable, non-

TABLE IV. Isolation of Toxic Factor by "Salting Out" with $(NH_4)_2SO_4$. Assayed by determining average survival time (in hr ± S.D.) of worms receiving 1 ml injections. Coelomic fluids were partially purified prior to saturation with $(NH_4)_2SO_4$ by autoclaving.

Partially purified toxic coelomic fluid saturated with $(NH_4)_2SO_4$		Partially purified normal coelomic fluid saturated with $(NH_4)_2SO_4$	
Precipitate (dialyzed)	Supernatant (dialyzed)	Precipitate (dialyzed)	Supernatant (dialyzed)
56 ± 13	Infinite	Infinite*	Infinite

* Only 8 worms injected.

TABLE V. Effect of Toxic Factor, Isolated by "Salting Out" with $(NH_4)_2SO_4$, on Cleavage of *Arbacia punctulata* Eggs. Coelomic fluids were partially purified by autoclaving, followed by centrifugation and dialysis.

	Partially purified toxic coelomic fluid saturated with $(NH_4)_2SO_4$		Partially purified normal coelomic fluid saturated with $(NH_4)_2SO_4$		
	Precipitate	Supernatant	Precipitate	Supernatant	Sea water control
	(Dialyzed)				
% of eggs cleaved	0	97	94	96	99

dialyzable toxic factor, capable of causing death when injected into normal worms. This toxic substance, originated from the cells of the coelomic fluid, has been partially isolated by saturation with ammonium sulfate. A semi-microbiological assay has been described.

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Reduction of Mitotic Activity in Pinna Epidermis of Mice Given Cortisol or 9α-fluorocortisol.*† (22343)

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The inhibitory effect of cortisone upon mitotic activity in certain tissues seems to be established(1-4). In this study, antimitotic effects were noted for hydrocortisone (cortisol) and for 9α-fluorocortisol. In order to detect antimitotic effects occurring within the range of the daily mitotic rhythm, this work was carried out 1) under environmental conditions standardized for evaluation of 24-hour periodicity(5), 2) during the period of day of relatively high mitotic activity, 3) on a population of mice previously found to exhibit a marked mitotic rhythm in pinna epidermis(6).

Materials and methods. For 7 days prior to experiment, male ZBC mice, 8 weeks of age, were kept singly housed in a room maintained at 80 ± 1°F, illuminated from 6 A.M. to 6 P.M., and kept dark from 6 P.M. to 6 A.M. Purina Fox Chow and tap water were available to the mice *ad libitum*, from the time of weaning and throughout experiment. On the day of experiment, a first group of 11 mice was not handled, while 3 other groups, each composed of 12 mice, were given intraperitoneally 100 γ or 1,000 γ of cortisol acetate or 10 γ of 9α-fluorocortisol acetate. Each mouse was weighed prior to injection and the dose was adjusted to refer to 20 g of body weight. The treatments were given in rotation, in order to minimize the possible role of a time factor difference among treatments.

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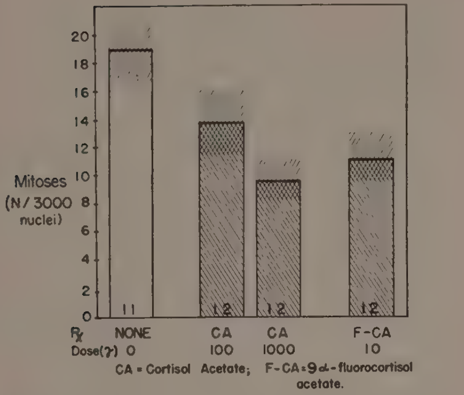

FIG. 1. Effects of cortisol and 9 α-fluorocortisol upon pinna mitoses of intact male ZBC mice (the height of the columns denotes the means, the shaded areas denote ± 1.0 S. E.).

TABLE I. Significance of Inter-Group Differences in Mean Mitotic Counts.

Difference studied*	DF	Difference* ± S.E.	t	P
9 α-fluorocortisol acetate 10 γ	21	7.7 ± 2.6	2.94	.008
Cortisol acetate 1000 γ	21	9.1 ± 2.3	3.90	<.001
" " 100 γ	21	4.8 ± 2.9	1.65	.115

* Mean mitotic count of "no treatment" group *minus* the corresponding value for treatment group listed.

The injections were made from 8:30 A.M. to 9:30 A.M. of the same morning, and the injected and uninjected mice were all sacrificed 4 hours later. The ears were clipped off by means of scissors, the margins were trimmed, and the ears were then placed into Bouin's fixative. The time elapsed from the moment of removal of a mouse from its cage until the immersion of its ear into the fixative did not exceed 10 seconds. Following embedding in paraffin, at least 20 sections of each ear were prepared. The sections, about 6 μ thick, were stained with Heidenhain's iron hematoxylin and were counterstained with eosin. In sections from each of the ears, 3,000 epidermal cells were examined under oil immersion and the total number of mitoses as well as their respective stages were recorded.

Results. The height of the columns in Fig. 1 shows the mean mitotic count for each of the groups studied, the shaded areas denoting ± one standard error. It may be seen that the mice given corticosteroid treatments had lower mean counts, as compared with untreated mice. As far as cortisol treatment is concerned, the group receiving 1000 γ had a lower mean count than that receiving 100 γ. Furthermore, the antimitotic effect of 10 γ of 9α-fluorocortisol compared favorably with the corresponding effect of 100 γ and unfavorably with that of 1000 γ of cortisol. The effects of both 1000 γ of cortisol and 10 γ of 9α-fluorocortisol were significant at the one percent level, as may be seen from Table I. The proportions of the 4 mitotic stages in the groups receiving either 10 γ of 9α-fluorocortisol or 1000 γ of cortisol were, however, roughly comparable to those in the untreated group, as shown in Table II.

The χ^2's for the slight differences in proportions of mitotic stages between the untreated group on one hand and the 10 γ of 9α-fluorocortisol and 1000 γ of cortisol groups, on the other hand, were 2.25 ($P>.39$) and 1.89 ($P>.57$), respectively. By contrast, the corresponding difference between the untreated group and the 100 γ of cortisol group was significant below the 5% level ($\chi^2 = 8.53$; $P = .037$). The latter group differs from the former groups primarily by a higher proportion of prophases and by a lower proportion of telophases. This difference in proportions may have been brought about by the shorter duration of the antimitotic effect of the lower dose of cortisol. Conceivably, in mice given 100 γ of cortisol, at 4 hours post-injection, cells may have entered prophase without inhibition, but as yet, they may not have ad-

TABLE II. Distribution of Mitotic Stages in Groups Investigated.

Treatment	Dose (γ)	% prophase	% metaphase	% anaphase	% telophase
None		26	43	11	20
9 α-fluorocortisol acetate	10	26	37	11	26
Cortisol acetate	1000	22	39	16	23
" "	100	33	44	13	10

vanced to telophase (Table II). This possibility, however, can not be supported without additional data obtained at earlier times after injection.

The main points of this report are the following: First, the administration of cortisol in 1000 γ doses, as well as that of 9α-fluorocortisol in 10 γ doses, results in a significant reduction in number of mitoses in pinna epidermis. Second, doses of the compounds which exhibit significant antimitotic effects do not change significantly the proportions of mitotic stages. Accordingly, these compounds may not interfere with the mitotic process *per se*, but rather, they may act upon some aspect of interphase. Third, a comparison of those doses of cortisol and of 9α-fluorocortisol which exhibit antimitotic activity supports the suggestion that the substitution of a fluorine atom in the 9α position results in a marked enhancement of the antimitotic activity of cortisol. The fluorine-substituted compound has more than 10 times the activity of cortisol, under the conditions of this study. This suggestion is in keeping with the enhancement of other biologic activities of cortisol by fluorine-substitution in the 9α position(7-13).

Summary. Mitotic activity in pinna epidermis of male ZBC mice was studied under environmental conditions standardized for evaluation of 24-hour periodicity, during the period of day of relatively high mitotic activity. Under these circumstances, antimitotic effects, exerted apparently during interphase, were noted for hydrocortisone and for 9α-fluorohydrocortisone. The latter compound had more than 10 times the antimitotic potency of the former.

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Plasma Enzyme Actvity in Myocardial Infarction in Dog and Man.* (22344)

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Increases of plasma transaminase(1) and lactic acid dehydrogenase in liver disease(2) and myocardial infarction(3) suggested the probability of the release of other enzymes from the myocardium or the liver as a result of hypoxia or tissue necrosis. This laboratory has been interested in the immediate changes following experimental myocardial infarction in dogs. For example, a higher concentration of pyruvate in coronary sinus than in arterial blood (negative myocardial balance of pyruvate) was observed within the first thirty minutes following coronary embolization(4). This phenomenon has also been observed in hemorrhagic shock(5) and in ventricular fibrillation(4). It is likely that the reduction in coronary flow which immediately follows

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embolization of the coronary arteries is responsible for the metabolic changes. Furthermore, myocardial ischemia leads to necrosis, with concomitant changes in cell permeability and loss of enzymes into the blood. The present report is concerned with variations in the plasma levels of transaminase, isomerase, aldolase, and malic acid dehydrogenase in dogs with experimental coronary occlusion and in man suffering from myocardial infarction.

Methods. Pyruvate was determined by the method of Friedemann and Haugen(6). Experimental coronary occlusion was produced by following essentially the method of Agress (7). However, human albumin was substituted for gum acacia as a vehicle for suspension of the plastic spheres (size of spheres: 325 mu; amount injected: 2 mg/kg weight). This modification was necessary since it was found that gum acacia alone can produce infarction with typical pathologic and biochemical alterations. Plasma transaminase activity was determined by the method of Karmen *et al.*(1) and plasma aldolase by that of Bruns (8); the latter is expressed as micrograms of triose phosphate formed per ml of plasma per hour at 38°C. Plasma isomerase activity was determined by the method of Bodansky(9) expressed in activity units as therein described. The determination of malic acid dehydrogenase in the presence of other unidentified enzymes and metabolites presents considerable difficulties. The reaction of malate, in the presence of DPN and malic acid dehydrogenase comes to a rapid standstill presumably due to accumulation of oxalacetic acid. The addition of glutamate, in the presence of excess transaminase removes this difficulty(10). Since, however, the reaction is greatly in favor of oxalacetate ⟶ malate(9) and involves only a few components, it was tested as follows: To 0.1 - 0.05 ml of plasma was added 2 ml of 0.1 M phosphate buffer pH 7.55 and 0.1 ml $DPNH_2$ (2.5 mg/ml); the volume was made up to 3.0 ml with distilled water. After 10-15 minutes 0.1 ml of 0.5 M oxalacetic acid (Sigma) at pH 7.0 in 0.1 M phosphate buffer was added. The change in optical density at λ340 of $DPNH_2$ was observed for 3 to 4 30-second periods to assay the reaction rate. The oxalacetate solution was prepared just before use and was kept in a glass stoppered test tube in ice water. In addition to decarboxylation reactions(11) which would result in some lactic acid dehydrogenase activity being measured simultaneously, oxalacetate can enter many

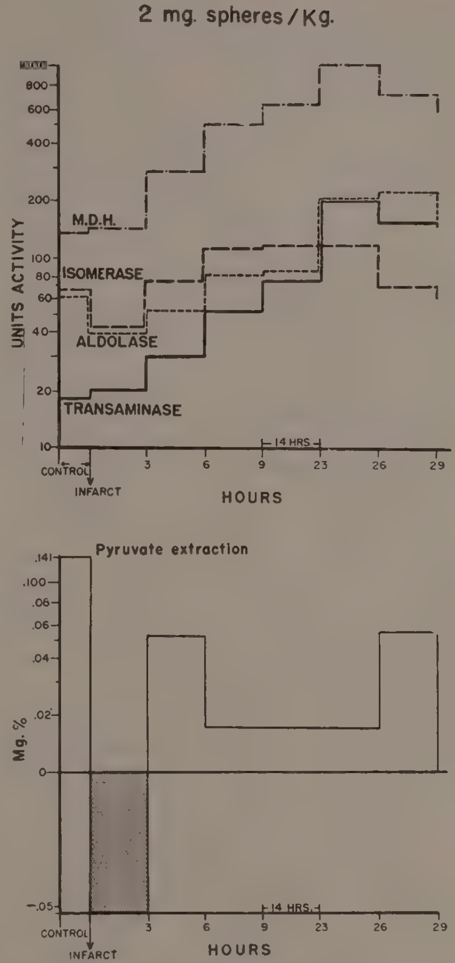

FIG. 1. Variations in plasma activity of malic acid dehydrogenase, isomerase, aldolase, and transaminase. Changes in plasma enzyme activity are correlated with myocardial pyruvate extraction. It may be seen that there is an immediate drop in myocardial pyruvate extraction; however, the negative myocardial balance is of short duration. Enzyme activity in plasma commences to rise 3 hr after coronary embolization. Peaks in plasma enzyme activity occur 24 hr following embolization.

other pathways(12). While a sharply rising activity with pH has been reported for lactic acid dehydrogenase(2) with a maximum at pH 9.0, the reaction described has an almost constant activity between pH 7.5 and 8.1. The range of normal activity units and the standard deviation are also less than that reported for lactic acid dehydrogenase(3). Since the reaction may not be entirely specific for malic acid dehydrogenase, it will be referred to in quotation marks.

Results. The average, range and standard deviation for the plasma concentrations of these enzymes in normal dogs are as follows: transaminase, 23, (10-37), 9; "malic acid dehydrogenase", 108, (42-195), 47; isomerase, 33, (16-68), 17; aldolase, 42, (24-68), 18.

Fig. 1 is representative of the results obtained after coronary embolization. In a series of 9 experimental animals, 7 were examined and showed postmortem evidence of myocardial infarction. Four animals of the experimental group were subjected to coronary sinus catheterization and showed negative myocardial pyruvate balances. In 3 of these animals myocardial pyruvate balances were negative within 15 minutes after the infarction and then became positive (Fig. 1). One showed a negative balance at 3 hours. It is seen from Fig. 1 that the significant release of enzymes began by the third hour. At 24 hours the increase in enzyme activity was maximal for all animals. At that time, the coronary flow and the coronary vascular resistance which had been diminished immediately following embolization of the coronary arteries had returned to their control levels. At 48 hours a rapid fall in plasma enzyme activity ensued. After 24 and 48 hours both transaminase and "malic acid dehydrogenase" showed increases of comparable degree, with aldolase and isomerase exhibiting increases of a lesser order.

Table I gives the normal values on a small series of humans without hepatic or cardiac disease. In man the average value, range and standard deviation for "malic acid dehydrogenase" and isomerase were less than those seen in the dog. Results obtained in patients with myocardial infarction are also illustrated in Table I. As compared to the dog, aldolase activity was least elevated in patients with myocardial infarction. Isomerase activity in plasma increased following in general the trend of transaminase. Similar findings were obtained with "malic acid dehydrogenase."

The high activity of "malic acid dehydrogenase" in normal plasma suggests the possi-

TABLE I. Levels of Plasma Transaminase, Malic Acid Dehydrogenase and Isomerase in Normals and Patients with Myocardial Infarcts.

		Transaminase	Malic acid dehydrogenase	Isomerase* (Bodansky units)	Aldolase as triose-phosphate formed per ml plasma per hr
Normals	Avg	25 (9)	79 (7)	13 (10)	26 (7)
	Range	15–39	50–104	6–19	21–32
	Stand. dev.	8	20	4	5
L.J.	2/ 5	185	333	111	59
	2/ 6	150	200	30	32
	2/ 7	47	163	15	25
	2/ 8	37	120	12	21
V.A.B.	2/ 6	155	300	51	41
	2/ 7	95	208	20	32
P.E.	1/30	123	200	94	65
P.P.	2/11	100	647	374	39
J.P.	2/11	34	240	54	24
	2/13	62	110	7	23
	2/14	43	152	9	24

* Normal values reported on a series of 87 patients by Bodansky: Avg, 20; range, 8-40; stand. dev., 8.

Figure in parenthesis represents No. of animals.

bility that the transaminase reaction could be run adequately without the addition of the expensive and labile malic acid dehydrogenase enzyme preparation. Omitting this enzyme has little effect on the results of the determinations on stored dog plasma, when the transaminase levels were at the limit or beyond the established normal range. However, when transaminase activity is determined in stored human plasma, the omission of malic acid dehydrogenase leads to results which are low and inconsistent.

The system used to determine "malic acid dehydrogenase" activity by means of the rate of oxidation of DPNH in the presence of oxalacetic acid and plasma may not measure malic acid dehydrogenase activity exclusively. Nevertheless, the rate of $DPNH_2$ oxidation appears to be closely related to the increase of activity of other enzymes, which are observed following myocardial infarction. The reaction appears to be particularly useful because of its simplicity and rapidity. Similarly, the isomerase determinations in myocardial infarction may be of clinical importance since they can be carried out without specialized equipment.

Summary. 1. Increased plasma levels of transaminase as well as aldolase, isomerase and "malic acid dehydrogenase" activity occurred in dogs after experimental myocardial infarction and in humans suffering from coronary occlusion. 2. Plasma levels of all enzymes were highest 24 hours after experimental myocardial embolization and had fallen rapidly at 48 hours. 3. Normal human values for plasma isomerase and "malic acid dehydrogenase" were significantly lower than those found in normal dog plasma. 4. In dogs following experimental myocardial embolization plasma transaminase, "malic acid dehydrogenase" and aldolase were increased to a greater extent than isomerase. In humans the plasma aldolase activity was comparatively less elevated than that of transaminase, "malic acid dehydrogenase" and isomerase. In both man and dog, coronary occlusion resulted in comparable increases of plasma transaminase and "malic acid dehydrogenase" activity. In dogs with coronary embolism, there was a reversible decrease in coronary flow myocardial oxygen usage, coronary vascular resistance as well as an early reversible negative pyruvate balance. At 24 hours the coronary flow and myocardial oxygen usage had returned to normal. 5. The negative myocardial pyruvate balance appeared to be an immediate response of an hypoxic myocardium. In contrast, the release of enzymes into the plasma was correlated with muscle necrosis. 6. A rapid and simple method is described for the determination of "malic acid dehydrogenase" activity in plasma.

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Metabolism of Acetoacetate-3-C^{14} by Mammalian Heart Muscle. (22345)

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In a previous paper(1) we have presented evidence indicating that the net increase of cardiac glycogen observed in ketonemic animals(2) does not represent a direct conversion of ketone bodies *per se* to carbohydrate. That demonstration invalidates the most obvious interpretation which can be applied to the data of Lackey *et al.*(2), and relegates the ketonemia to some secondary role yet to be defined. While our data was direct and conclusive, the subject is one of sufficient importance to require evaluation from a different viewpoint before definitely ruling out the possibility that cardiac tissue may have some unique metabolic mechanism for dealing with ketone bodies.

In the present paper we have used the isolated dog heart to examine the metabolic path traversed by acetoacetate-3-C^{14} as reflected by the isotope distribution in the endogenous malate and succinate isolated at the end of the experiment to serve as an internal metabolic indicator. Glycogen is frequently employed in this capacity(3) but was valueless here because no tagging of that molecule occurs under the conditions used, an observation which is consistent with our *in vivo* experiments(2).

Materials and methods. The isolated dog heart was used in a simple glass apparatus containing the usual components and through which the heparinized blood (500 ml in all experiments) was circulated by means of a manual "rubber bulb-check valve" type of pump. The gas outlet from this closed system was led through 2 refluxing setups for trapping acetone in boiling acid mercuric sulfate (4) and thence to an efficient alkali CO_2 absorbing tower. A slight vacuum was used to overcome the mild resistance to the free flow of gas through the system. *Sodium acetoacetate-3-C^{14}* was synthesized and prepared by established methods(5). Five millimoles of the radioactive compound was introduced dropwise into the flowing blood just as it entered the marble filled gassing tower. The 500 ml of circulating fluid was thus 0.01 M with respect to acetoacetate. Our experiment made use of several different gases: 100% O_2, 95% O_2-5% CO_2 and, in a few instances, a gas mixture calculated to simulate the oxygen tension found *in situ* and composed of 19% O_2-5% CO_2-76% N_2. With the first 2 gases we used undiluted blood in the apparatus and obtained a very satisfactory heart preparation. With the latter mixture however, the blood did not appear well oxygenated and quickly assumed a marked viscosity with resultant limitation in coronary blood flow, and feeble heart action. In these instances we diluted the blood 1:1 with Ringer-bicarbonate in order to maintain an adequate fluid flow. Even then the preparation was not nearly as vigorous as at the higher oxygen levels. At the end of a 2 hour experimental period, 2 slightly different approaches were used to isolate the malate and succinate present in the heart tissue. In one instance we followed directly the procedure outlined by Needham(6) after adding 0.2 mM of succinate or malate carrier. In the other we introduced more drastic conditions to assure release of any tissue bound dicarboxylic acids, or those that might perhaps be tied up in some sort of ester linkage. Using a Waring Blender, the cardiac tissue was homogenized in ice water (carrier succinate added) and made to pH 2 with concentrated sulfuric acid. The entire material was steam distilled, collecting ten volumes of distillate, and the resulting tissue suspension reduced to an almost dry paste at mild temperatures using a blast of dry air. After adding concentrated sulfuric acid and dehydrating with plaster of Paris the succinate was recovered and purified(6). Occasionally we effected further purification by use of partition chromatography on silicic acid(7). Our results were not affected by the method of extraction. Carbon-wise degrada-

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TABLE I. Isotope Distribution in Malate and Succinate following Metabolism of Sodium Acetoacetate-3-C^{14} by Isolated Dog Heart.

Gas	Other additions	Total C^{14} added,* cts/min. (×1000)	$C^{14}O_2$ in respiratory CO_2†	% substrate to CO_2	Specific activity, malate cts/min./mg/C Carboxyl C	Specific activity, malate cts/min./mg/C Methylene C	Specific activity, succinate Carboxyl C	Specific activity, succinate Methylene C
O_2	None	2,626	703,000	26.7	683	0		
19% O_2–5% CO_2–76% N_2	"	4,150	791,420	19	2,553	17	1,748	58
Idem	Adrenalin	4,150	337,777	8	2,026	10		

* As sodium acetoacetate-3-C^{14}.

† Blood acidified at end of experiment and bound CO_2 aerated using N_2.

tion of the isolated succinate or malate (and occasionally both) was effected by established methods(8) giving the activity respectively in the carboxyl and methylene carbons.

Results. According to current concepts, acetoacetate-3-C^{14} is metabolized by first splitting into 2 "acetate" molecules one of which, in this case, would be labeled in the carboxyl group. Further breakdown would be effected by traversing the tricarboxylic acid cycle and producing among the various other components, succinate and malate labeled exclusively in the carboxyl groups(3). For each "acetate" entering the cycle 2 carbons are lost as CO_2 and this mechanism therefore cannot account for a net increase in any component. If the results of Lackey *et al.*(2) represent a direct relation between ketone bodies and net carbohydrate synthesis, then a path not involving the tricarboxylic acid cycle must be invoked. This could happen, for example, if omega oxidation converted acetoacetate directly to oxalacetate and thence to glucose via pyruvate. In this instance 3 of the 4 initial carbons would be available for the net synthesis. Another possibility involves the reduction of acetoacetate to butyrate and thence to succinate via omega oxidation and finally to glucose as in the above case. The latter is unlikely in view of Lorber's(9) recent demonstration that butyrate is metabolized only in the conventional manner, an observation which we have confirmed in the isolated dog heart(10). If either of the latter mechanisms is operating on acetoacetate-3-C^{14} the endogenous malate and succinate would be tagged in the methylene carbons.

Table I gives data which are typical of our various experiments. Only comparison between the activity in the carboxyl *vs* methylene carbons should be made. The absolute specific activity of the several samples is not comparable because the amount of carrier dicarboxylic acid added to aid in the isolation procedure varied according to circumstances. It is obvious that in comparison to the carboxyl groups, the methylene carbons of the malate and succinate contain an insignificant amount of C^{14}. This is consistent with the view that acetoacetate is being metabolized only in the established manner, *viz*, by breakdown to two two-carbon fragments which then traverse the tricarboxylic acid cycle(3), a path which cannot effect a net increase of any component.

We have made additional observations that seem worth recording. We were curious to assay the possibility that the heart may contain dormant alternate metabolic paths on which it can draw in an emergency. Accordingly, we stimulated a preparation to abnormally vigorous activity by the periodic addition of adrenalin to the circulating blood. As shown in Table I the isotope distribution in malate was not altered. Whether the low total conversion to $C^{14}O_2$ (8%) is a function of the adrenalin or merely a reflection of variability from preparation to preparation is a matter under consideration.

Summary. The metabolic path of acetoacetate-3-C^{14} in the isolated dog heart has been determined using the isotopic distribution in the endogenous malate and succinate as an internal metabolic indicator. Isotopic carbon was found exclusively in the carboxyl

groups of malate and succinate as would be expected if acetoacetate was being utilized only by the established path (acetoacetate → 2 "acetate" → 4 CO_2 via the tricarboxylic acid cycle). This is taken as corroborative evidence for our previous conclusion that ketone bodies *per se* are not responsible for the net increase in cardiac glycogen noted in ketonemia.

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Effect on Cardiac Glycogen of Intravenously Administered Sodium Acetoacetate-3-C^{14}. (22346)

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In dogs made diabetic by pancreatectomy the glycogen content of liver and skeletal muscle decreases while that of heart increases (1). A similar pattern has been observed in rats injected with large amounts of acetoacetate or B-hydroxybutyrate(2). It becomes a matter of some importance to establish whether the ketone bodies *per se* are being metabolized in a manner directly responsible for this net increase in cardiac glycogen or are merely acting in some secondary role. In the former instance a new and yet unrecognized mechanism would have to be operating, since the conventional metabolic path (acetoacetate → 2 "acetate" → 4CO_2 via the tricarboxylic acid cycle) is not compatible with a net increase in any component. If ketone bodies on the other hand are acting only in some indirect manner, that role too must be defined. The data published by Lackey *et al.* (2) simply notes the quantitative increase of cardiac glycogen without throwing any light on possible mechanisms. The present paper is the first in a series designed to define this particular metabolic area. Here we have made the direct test of whether ketone bodies *per se* go to heart glycogen. We have injected into rats large amounts of acetoacetate-3-C^{14} and subsequently isolated the cardiac glycogen to determine the quantity and distribution of C^{14} in the glucose molecule. By this means strong inferences concerning the metabolic path can be obtained.

Materials and methods. Sodium acetoacetate-3-C^{14} was synthesized according to established methods(3) and put into a solution at pH 7.4 such that per ml there was 0.88 mM (11%) of acetoacetate containing an amount of C^{14} giving 600,000 cts/min. The quantity of isotopic material available limited the study to 3 rats. The nature of the results is such that valid conclusions are clearly possible despite the small number of animals involved. A 200 g rat, warmed by a heat lamp, was anesthetized with nembutal, the jugular vein exposed, and the fine cannula of an intravenous drip apparatus introduced. In each of our 3 experiments the gravity flow ceased after about 2½ hours at which time 3 ml (2.6 mM and 1,800,000 cts min.) of the acetoacetate solution had been introduced. In one rat we used a hypodermic syringe to inject an additional 2 ml into the tail vein over a period of half an hour. In all instances a total of 4 hours elapsed before the animal was sacrificed. Following this, the heart was removed, washed with saline and dropped into hot 30% KOH together with 35 mg of purified glycogen made from rat liver. The carrier material was necessary in view of the fact that a single rat heart contains only about 2 mg of glycogen which is an inadequate amount for isolation, purification and degradation. The dilution does not interfere with evaluation of radioactivity since counting was done on the total sample which was highly purified (4), degraded to CO_2 by persulfate oxidation (5) and assayed as $BaC^{14}O_3$.

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Results. According to Lackey *et al.*(2) the infusion of acetoacetate can cause as much as a 60% increase in heart carbohydrate. In the average rat this represents formation of approximately 1.5 mg of glycogen over and above that normally present. We would not expect our experiments to effect this much synthesis since we introduced only about 60% as much ketone as the original workers. However, assuming that we obtained only 10% as much synthesis and assuming that no more than one C^{14} from the acetoacetate was incorporated into each glucose of the glycogen molecule, we would have expected radioactivity in the order of 500 counts per minute. In none of our experiments did the glycogen molecule contain any traces of radioactivity. We are forced to conclude therefore, that the increase in heart glycogen accompanying ketonemia does not represent a direct conversion of ketone bodies to carbohydrate. The mechanism of this effect is still an open question.

Summary. The intravenous injection of large amounts of acetoacetate-3-C^{14} has been effected under conditions reported to cause a net increase in heart glycogen. Isolation and degradation of the cardiac glycogen failed to divulge any trace of radioactivity. It thus appears that the net increase in heart carbohydrate accompanying ketonemia represents some secondary and yet undetermined influence of ketone bodies.

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Feasibility and Safety of Frequent Plasmapheresis of the Same Human Donors.* (22347)

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Plasmapheresis is the obtaining of whole blood from a donor with the retention of the plasma; the red blood cells being returned to the donor. This has been accomplished in the past in both man and other animals by well known technics(1-5). These are laborious, time consuming, and have many other obvious drawbacks inherent in the methods which would prevent large scale use. The present study is concerned with plasmapheresis in man utilizing the ADL-Cohn Blood

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Fractionator.[†] The theoretical aspects of this technic have been discussed(6). With this equipment the red blood cells and plasma are continuously separated during the phlebotomy. It provides a sterile closed mechanical system for taking blood from a donor and separating it into many of its constituents, any of which may be returned immediately to the donor through the same needle used for the phlebotomy. The temperature of the removed blood during separation is decreased to 8°C or lower in a very short period of time which prevents adverse enzyme activity. Completely non-wettable surfaces have been utilized throughout, consisting of either polyvinyl plastic[‡] or silicone-coated[§] metal. A complete description of the Blood Fractionator is being prepared(7). One of the problems being investigated in the present study is to determine whether biweekly plasmapheresis of the same donor over a period of one year is safe and feasible. This project has been in progress since April 1955 (about 40 weeks) and because of the practical applications, as well as the broad theoretical implications, it seemed advisable to present a preliminary report.

Methods and materials. Plasmapheresis technic. The donor lies on a table placed on a raised platform which, by gravity, increases the rate of flow of blood to the Fractionator which is about 40 inches lower. The phlebotomy is started with the insertion of a silicone-coated 15 gauge needle into a larger vein, usually in the antecubital fossa. The blood flows through a 3 way stop cock[‖] and through an ion exchange resin column[¶] which removes the calcium and some of the platelets. The decalcified blood then continues into the centrifuge bowl, which for this operation is designed to accept a fixed volume of 500 ml of fluid for each run. While the bowl is spinning, only the plasma (the lighter component) is allowed to flow from the bowl into a plastic bag. No admixture of red blood cells occurs. The red blood cells, which are heavier, accumulate within the bowl and flow by gravity, after the bowl is stopped, into another plastic bag. The plastic tubings, through which the plasma and red blood cells respectively flow to the bags and which are connected to the bowl are then sealed[**] (a one-half inch wide seal), and the seal is cut through the middle with a pair of scissors. This operation thus produces sterile containers of plasma and red blood cells with short lengths of attached tubing sealed at the end. Red blood cells are returned to the donor by connecting the plastic tubing attached to the red cell bag, to the 3 way stop cock. Incorporated in the plastic tubing outlet of the red cell bag is a drip chamber and a nylon mesh filter. In the 3 to 4 minute period during which the red cells drop from the centrifuge into the bag, sterile physiological saline is slowly infused into the donor in order to keep the lumen of the needle and tubing open for the return of the red blood cells. To some extent, before the red cells are returned, the saline replaces temporarily the volume of the whole blood removed. *Donors.* Twenty-three men are being utilized in this study. Nineteen of these volunteers are members of the Philadelphia Park Police between the ages of 26 and 35, in excellent physical condition. The remaining 4 men are between 37 and 43 years of age. *Tests.* The following determinations are made at each visit: Blood pressure, pulse, red blood cell count, white blood cell count, differential counts, platelet count, hemoglobin, hematocrit, sedimentation rate and prothrombin clotting time. In addition, paper electrophoretic pattern and nitrogen content are determined for each plasma.

Results. Table I shows that there is very little variation in the red cell count, hemoglobin, hematocrit and plasma nitrogen in a single donor subjected to biweekly plasmapheresis over a period of 40 weeks. In addition the amount of time for each phlebotomy and red blood cell return is recorded. These data, pertaining to a single donor, are representative of the entire group of 23 individuals in the study.

White blood cell counts, differential counts,

[†] Mfd. by A. D. Little, Inc.

[‡] Mfd. by Fenwal Labs., Inc.

[§] Mfd. by General Electric.

[‖] The 3 way stop cock is being replaced by a plastic Y tube.

[¶] Dowex 50.

[**] Mfd. by Scientific Specialties Corp.

TABLE I. Effect of Biweekly Plasmapheresis on the Same Donor over a Period of 40 Weeks.

	Weeks										
	0	4	8	12	16	20	24	28	32	36	40
Red blood cell counts (× 10^6)	4.7	4.9	4.6	4.5	5.6	5.2	4.9	5.4	5.1	4.7	4.9
Hemoglobin (g)	15.8	15.2	15.1	16.3	15.4	15.0	15.7	15.0	15.8	14.9	14.5
Hematocrit (%)	42	40	41	38	40	40	40	43	45	43	46
Plasma N (%)	1.07	1.14	1.00	1.02	1.08	1.00	1.03	1.01	1.04	1.08	1.06
Phlebotomy time*	7 35	9 45	9 00	7 40	7 46	10 02	7 21	7 40	15 10	7 23	7 40
R.B.C. return time*	7 50	10 10	9 12	12 35	9 40	7 05	8 30	9 08	11 12	10 57	9 34

* First number in these columns is minutes, last 2 numbers are seconds; *e.g.*, 7 35 = 7 min. 35 sec.

Figures on alternate biweekly plasmaphereses are omitted because of lack of space.

platelet counts, sedimentation rates and prothrombin clotting times carried out routinely at each donor's visit have been quite constant, much the same as would be found in any normal person.

To make this technic a practical procedure, the time consumed must be relatively short. The average time required for the phlebotomy and red blood cell return of the 23 men for 20 plasmaphereses (a total of 460) is 8′ 50″ and 10′ 04″ respectively. Allowing 3 minutes for the red cells to drop after the centrifuge has been stopped, the total time elapsed is 8′ 50″ + 10′ 04″ + 3′ = about 22 minutes. It is a simple matter to run 3 complete plasmaphereses per hour by beginning a phlebotomy on the second donor after the red blood cells are started in return to the first donor.

Over 550 plasmaphereses have been carried out since the start of this project including the preliminary and trial runs. No untoward reactions have been observed in any donor.

Discussion. The utilization of the new equipment mentioned has made frequent plasmapheresis of the same human donors both safe and feasible. This permits the use of donors for plasma only, with the replacement of their red blood cells, thus allowing 26 bleedings per year in this study. Fifty plasma donations yearly should be perfectly feasible since the human plasma proteins are regenerated rapidly(1,3), and since a very small percentage of the total plasma proteins is removed at one time. (200 ml plasma x 7.5% protein equals 15 g of protein out of a total plasma protein content of about 250 g, or about a 6% removal at one time.)

If 10 times the customary number of donations can be obtained from a single individual, it should be possible to achieve large savings in the collection of plasma since only one-tenth the number of donor centers would be required.

By plasmapheresis, single plasmas, as free of bacteria and hepatitis virus as the usual single donation of whole blood, can be preserved in non-porous bags, easily stacked and non-breakable, which can be left at any desired temperature for the inactivation of serum hepatitis virus. This virus is probably present in 0.5 to 1% of individual bleedings from the general population, but with selected donors for repeated plasmapheresis the incidence of virus in single plasmas should be considerably less than in whole blood. The possibility of untoward reaction in the recipient, to certain plasmas could be obviated by testing the individual plasma before use.

The plasmapheresis technic makes feasible the use of human donors in the preparation of high titered specific antibodies for therapy. The danger of foreign protein reaction is practically eliminated. Furthermore, it has been found that, unit for unit, the ratio of effectiveness of human antibody is far superior to horse antibody(8). As part of the current project the persistence of viral and bacterial antibodies in hyperimmune and other donors undergoing biweekly plasmapheresis is being determined. A further use of this method would be to obtain relatively large amounts of plasma from individuals who have rare antibodies for a short period of time, *e.g.* mothers following delivery, who have certain Rh or Hr antibodies which decrease in titer fairly rapidly.

Summary. Biweekly plasmapheresis on the

same human donors over a period of 40 weeks has been found to be feasible and safe. The possibilities for obtaining large amounts of concentrated human antibodies and for stockpiling sterile human plasmas are discussed.

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Failure of Ovarian Hormones to Maintain Pregnancy in Rats Deficient in Pantothenic or Pteroylglutamic Acid.* (22348)

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Recent studies have shown that reproductive failure, *i.e.*, fetal death and resorption, can be prevented and pregnancy maintained by daily injections of estrone and progesterone in rats subjected to 4 different dietary deficiencies, those of thiamine(1), pyridoxine (2), protein(3,4) or potassium(5). The daily dosage levels of the hormones were the same as those found to be effective in maintaining pregnancy in rats hypophysectomized and oophorectomized after breeding(6). The present communication reports the failure of these hormones to prevent fetal death and resorption in rats deficient in either pantothenic acid(7) or pteroylglutamic acid(8,9).

Methods. The procedures for testing the efficacy of the hormones were the same as those used previously to maintain pregnancy in other dietary deficiencies(1-5). The experimental conditions necessary for each vitamin deficiency to result in fetal death and resorption in approximately 90% of the animals were determined, if not already known. The synthetic hormones were dissolved in sesame oil and given daily, either separately or in combination, by subcutaneous injection from the 3rd or 5th days of gestation period to the day before autopsy. Levels of estrone varying from 0.5 to 2.0 μg[†] daily were given separately and levels from 0.5 to 1.0 μg were given in combination with 4 mg of progesterone. Five to ten rats (Long-Evans strain) were used for each experimental group. Vaginal smears were examined daily for the presence of erythrocytes, the sign of implantation. The rats were autopsied near the end of the gestation period, from the 15th to the 21st day. The basal (control) purified diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated vegetable oil (Crisco or Primex), and 4% salts no. 4(10). Crystalline vitamins per kilo of diet were: 300 μg d-biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 5.5 mg pteroylglutamic acid, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg

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[†] Maintenance of pregnancy has been shown to vary with level of estrone given to protein-deficient rats(3).

TABLE I. Failure to Maintain Pregnancy by Injections of Estrone and Progesterone in Pantothenic Acid-Deficient Rats.*

Daily hormone dosage	Rats bred (No.)	Wt change during gestation (g)	Onset vaginal RBC (day)	Resorptions (%)	Litters (%)
Pantothenic acid-deficient diet					
None	15	+ 5	12.4	79	21
Estrone 1 μg	6	+14	12.2	83	17
Progesterone 4 mg	10	+ 3	12.2	80	20
Estrone .5 μg + Progesterone 4 mg	10	− 3	12.1	80	20
Pantothenic acid-supplemented diet					
None	10	+115	13.1	0	100

* All groups averaged 25-30 days of deficiency prior to breeding. Control group received pantothenic acid-supplemented diet only from day of breeding to autopsy (day 17-18). Hormone injections were given daily from day 5 to autopsy.

niacin, 50 mg d-calcium pantothenate, 400 mg inositol, and 1.0 g choline chloride. All rats received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vit. A, 115 chick units vit. D, 6 mg dl-alpha-tocopherol, and 650 mg corn oil (Mazola). Omission of calcium pantothenate from this diet resulted in a pantothenic acid-deficient diet similar to that used previously(7) but improved in vitamin supplementation. In the PGA-deficient diet PGA was omitted and 1% succinylsulfathiazole and 0.5% x-methyl PGA incorporated in the diet. Fifty mg PGA per kilo of diet (10 times the usual level) was added to this diet for control animals. The PGA-deficient and supplemented diets are identical with those used previously(8,9).

Results. Pantothenic acid deficiency. Normal female rats from the stock colony were placed on the pantothenic acid-deficient diet at 90 to 100 days of age and bred after 25 to 30 days of deficiency. Hormone injections were given from the 5th day of gestation to the 17th or 18th days, when all animals were autopsied. Table I demonstrates the failure to maintain pregnancy by estrone and

TABLE II. Failure to Maintain Pregnancy by Injections of Estrone and Progesterone in Rats Subjected to Pteroylglutamic Acid (PGA) Deficiency.*

Daily hormone dosage	Rats bred (No.)	Wt change during gestation (g)	Onset vaginal RBC (day)	Resorptions (%)	Litters (%)
PGA-deficient diet instituted day of breeding					
None	10	+15	12.5	100	0
Estrone 1 μg	5	+31	13.0	100	0
Progesterone 4 mg	5	+14	12.8	100	0
Estrone 1 μg + Progesterone 4 mg	5	+32	12.8	100	0
PGA-deficient diet instituted day 9					
None	22	+62	12.8	100	0
Estrone 2 μg	10	+62	12.6	100	0
Estrone 1 μg + Progesterone 4 mg	10	+71	12.6	90	10
PGA-supplemented diet instituted day of breeding					
None	5	+ 58	12.0	0	100
	21	+112	12.8	0	100

* Hormone injections were given daily from day 3 to autopsy when PGA-deficient diet was instituted on day of breeding but were not given until day 9 in 2nd group when PGA-deficient diet started day 9.

progesterone in these deficient rats. Resorption occurred in approximately 80% of the animals, whether uninjected or given the ovarian hormones, either separately or combined. In all groups vaginal erythrocytes (RBC) appeared normally on the 12th to 13th days. The animals showed no signs of the deficiency with the exception of a failure to gain weight during the gestation period.

Pteroylglutamic acid deficiency. Stock female rats were bred at 90 to 100 days of age and placed immediately on the PGA-deficient diet. Injections of the hormones were started 3 days after breeding and all rats were autopsied on the 15th to 16th day of gestation. Table II shows the ineffectiveness of the ovarian hormones in maintaining pregnancy. Resorption occurred in all animals, regardless of the presence or absence of injected hormones. A less severe PGA-deficiency was then produced by instituting the PGA-deficient diet 9 days after breeding, a procedure previously reported to result in 100% resorptions(9). Hormone therapy was instituted at the same time as the PGA-deficient diet. Again the hormones were ineffective as fetal death and resorption occurred in 90-100% of the animals in all groups.

In all PGA-deficient rats vaginal erythrocytes occurred normally on the 12th to the 13th days and the animals exhibited no external signs of the deficiency, although the maternal weight gain during the gestation period was less than that of vitamin-supplemented controls.

Discussion. The failure to maintain pregnancy by injection of ovarian hormones in this study was in marked contrast to the successful maintenance of pregnancy with the hormones in deficiencies of thiamine, pyridoxine, protein or potassium. Under the experimental conditions reproductive failure resulting from pantothenic acid or PGA deficiency did not appear to be due to lack of production or secretion of the maternal sex hormones as has been indicated for the other four dietary deficiencies. The signs of hormonal inadequacies observed in rats subjected to the other four dietary deficiencies or in rats hypophysectomized and oophorectomized after breeding, namely early appearance of vaginal erythrocytes and extremely rapid resorption of implantation sites, were not observed in rats deficient in pantothenic acid or in PGA. There were no disturbances in the estrous cycle in unbred rats given the pantothenic acid or PGA-deficient diets for the same length of time. In this study the maternal organism was only slightly affected, few or no external signs of either deficiency syndrome being observed, even though fetal death resulted. The deleterious effects of both pantothenic acid and PGA deficiencies on embryonic development are well established as multiple congenital abnormalities have been observed in the offspring when a less severe deficiency was employed(9,11-13). In contrast, congenital abnormalities have not been reported for those deficiencies in which pregnancy can be maintained by injections of the ovarian hormones.

Summary. Daily injections of estrone and progesterone, given separately or combined, failed to prevent fetal death and resorption in rats deficient in pantothenic acid or in pteroylglutamic acid. Dosage levels of the hormones were the same as those used successfully to maintain pregnancy in rats hypophysectomized and oophorectomized after breeding as well as in rats deficient in thiamine, pyridoxine, protein or potassium. Under the experimental conditions, reproductive failure in rats subjected to pantothenic acid or PGA-deficiency did not appear to be due to lack of the maternal sex hormones.

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Improved Urinary Excretion Test for Assay of Intrinsic Factor. II. Sensitive Counting Technic.[*] (22349)

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The measurement of urinary excretion of vit. $B_{12}Co^{60}$ ($B_{12}Co^{60}$) following oral administration is used in the diagnosis of pernicious anemia(1,2) and as an assay for intrinsic factor(3,4). The use of human beings as experimental subjects necessitates the use of $B_{12}Co^{60}$ of low activity.[†] Since Co^{60} is a gamma emitter, the instrument of choice for its measurement is the scintillation counter. Most present well-type scintillation counters however, cannot count more than a 10 ml sample and therefore the low activity present in a 24 hour urine sample presents a counting problem which can only be solved by excessively long counting or by concentration of the urine samples. Flat scintillation crystals, upon which a beaker of liquid can be placed, do not offer significantly greater sensitivity than the well-type scintillation counter. Weiner and Peterson(5) designed a G-M well counter for measuring the radioactivity in liquid samples contained in 3 liter bottles, whereas Baskin *et al.*(6) constructed a well-shaped scintillation counter with a 35 ml capacity.

Using an available small well-type scintillation counter, a beaker with a capacity of one liter was designed fitting into the well and surrounding the top and sides of the crystal. This paper reports on the results obtained with the use of this beaker.

Methods. The design of the scintillation counter[‡] is shown on the left side of Fig. 1. The counter consists of an RCA 5819 phototube, a thallium activated sodium iodide crystal, 1¾ in. diameter and 1 in. thick with a hole ¾ in. in diameter and 1 in. deep, and a cathode follower preamplifier. A 4 ml sample can be counted in a screw-cap glass vial and the vial is then placed in the well separated by a brass sleeve to prevent contamination of the crystal housing. The beaker in counting position is shown on the right side of Fig. 1. It was constructed[§] starting with a two liter beaker from which the bottom was removed. The new bottom enclosed the crystal on top and sides with a projection fitting downward into the well. The counter and beaker were enclosed in a lead shield 3 in. thick. The background was about 110 cpm.

Results. 0.5 μc of vit. $B_{12}Co^{60}$ was diluted to 1000 ml and increasing amounts of this solution were placed in the beaker and counted. The values reported in Table I are corrected for background. As is evident from Table I, the use of the beaker gives an increase in counting rate of approximately 40 times that obtained using the standard 4 ml vial. As one would expect, the efficiency of counting with the beaker (3.5%) is less than direct counting in the vial (22%), but the over-all gain due to the larger volume is much greater than the loss in efficiency. It is reasonable to expect that much greater sensitivity would result if the beaker was designed to fit over larger crystals such as the commercially available 1¾ in. diameter by 2 in. thick crystals.

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[*] See Ref. 4 for Paper I in this series.

[†] The maximum permissible dose has been set at 3 μc by the Subcommittee on Permissible Internal Doses of the National Committee on Radiation Protection.

[‡] Model S_1—Purchased from H. O. Anger, Berkeley, Calif.

[§] Constructed by L. & K. Glass Co., Lyndhurst, N. J.

Due to the geometry of this beaker and crystal a volume of 500-750 ml gave the greatest efficiency, since at this volume there is equal amounts of liquid equidistant from all surfaces of the crystal. The optimum method, though, is to count one liter of liquid if available.

The increase in sensitivity permits the use of $B_{12}Co^{60}$ of lower specific activity in the urinary excretion test. One subject can receive 12 doses of 0.25 μc instead of 6 doses of 0.5 μc therefore, allowing the use of the same patient for additional intrinsic factor assays. Furthermore, the use of the beaker requires less counting time with a low excretion of radioactivity and makes it unnecessary to concentrate the urine sample. Some of the advantages of the beaker over the vial methods are listed in Table II. Even at the 5.0% excretion value if the dose of $B_{12}Co^{60}$ administered were reduced to the same amount as permitted by the use of the beaker and if the urine were not evaporated it would be necessary to count 100 minutes in order to reduce the standard error of counting to below 5%.

The technic described in this report also appears applicable to other biological materials of large volume and low activity from gamma radiation. It would seem particularly suitable for counting $B_{12}Co^{60}$ in feces in

TABLE I. Comparison of Sensitivity of Vial *vs.* Beaker Counting Methods.

Vol	C.P.M.	Activity in sample, μc	Efficiency, %	Beaker sensitivity ratio*
Vial				
4	978	.002	22	—
Beaker				
100	3,783	.05	3.4	3.9
200	7,832	.10	3.5	8.0
300	12,695	.15	3.8	13.0
500	21,769	.25	3.9	22.2
750	32,492	.375	3.9	33.3
1000	38,666	.50	3.5	39.8

$$^{*}\ \frac{\text{Efficiency} \times \text{volume of beaker}}{\text{Efficiency (22\%)} \times \text{volume of vial (4 ml)}}.$$

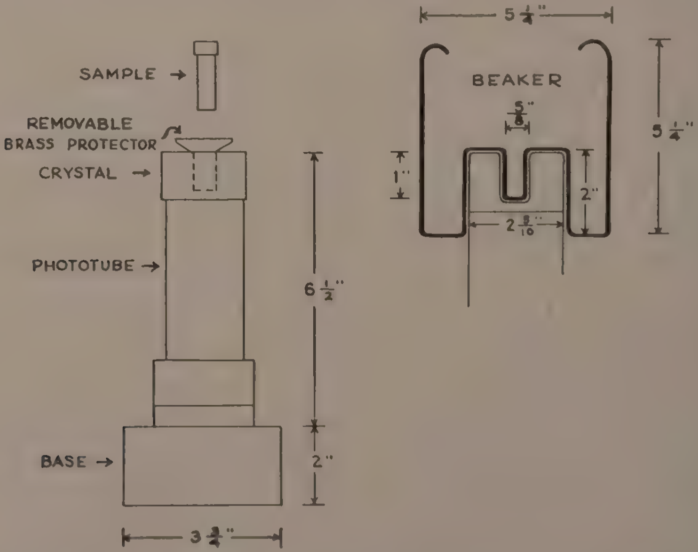

FIG. 1. Left: Well-type scintillation counter for measuring gamma radiation contained in a 4 ml glass vial. Right: Same counter showing design and position of a beaker for counting up to one liter of liquid.

TABLE II. Comparative Features of Vial and Beaker Counting Methods.

Excretion in 24 hr urine sample*	0.5%		5.0%	
	Vial	Beaker	Vial	Beaker
Dose of $B_{12}Co^{60}$ administered (μc)	.5	.25	.5	.25
Max permissible No. of doses/patient	6	12	6	12
Degree of concentration	10	None	10	None
Vol counted (ml)	4	1000	4	1000
C.P.M. corrected for background	49	97	490	970
Counting time† (min.)	20	9	1	1

* Assuming the 24 hr urinary vol to be 1 liter.

† Minimum necessary time so as not to exceed 5% stand. error of counting and assuming a constant background rate of 110 cpm.

the fecal excretion technic for the estimation of intrinsic factor activity(7).

Summary. Measurement of gamma radiation of low specific activity contained in relatively large volumes can be adequately performed with the use of a specially designed beaker which fits into the well and covers the cross section of a well-type scintillation counter. The increase in sensitivity obtained with this beaker allows the administration of smaller oral doses of vit. $B_{12}Co^{60}$ for additional assays using the same patient for the urinary excretion tests for intrinsic factor. In addition, no concentration of the urine sample is necessary and less counting time is required.

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Effect of Nalorphine on Gastric Emptying in Man. (22350)

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The consensus of most authorities is that morphine noticeably retards the emptying of the stomach in the human(1-3), as well as in the dog(4). Nalorphine, which antagonizes the narcotic effect of morphine when both are present, has certain actions when given by itself similar to morphine, but less intense. It is, therefore, of interest to determine the effect of nalorphine alone on gastric emptying.

Methods. The experiments were performed on 7 healthy medical students, 5 males and 2 females. The test meal consisted of 15 g of farina, cooked to a volume of 200 cc. One hundred g of barium sulfate were added so that the meal could be seen with the fluoroscope. The subjects ate this meal about 8:30 A.M. No food had been eaten since the previous evening. Determinations were made at weekly intervals. Fifteen minutes before they ate the meal, the subjects were given a subcutaneous injection of either saline (controls) or 5 mg of nalorphine hydrochloride* in saline. The time it took the meal to leave the stomach was determined fluoroscopically to the nearest 15 minutes.

Results. Table I shows that nalorphine delayed gastric emptying in all subjects, and more than 4 hours in 2. Only two showed a delay of less than 2 hours. All subjects, ex-

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* We wish to thank Merck and Co. for the gift of nalorphine hydrochloride used.

TABLE I. Effect of Nalorphine on Gastric Emptying Time of 7 Subjects.

Controls		Experimental			
No. of trials	Avg emptying time, hr	No. of trials	Avg emptying time, hr	Diff.	p[*]
3	2.67	3	6.83	4.16	.03
4	2.44	3	5.17	2.73	<.01
4	2.25	3	2.83	.58	.10
4	2.06	4	2.81	.75	.05
3	2.08	3	6.42	4.34	.02
3	2.00	2	5.25	3.25	.09
3	3.08	3	6.00	2.92	.01
Avg	2.37		5.04	2.67	<.01

[*] p according to the Fisher t test.

cept No. 3 and 6, showed a statistically significant delay, and in No. 6 lack of significance is possibly due to the small number of trials since he showed a large difference. The average delay for the series as a whole is significant at the one per cent level.

Discussion. Initially, a dose of 10 mg was tried on 4 of the subjects, but this produced severe malaise, and since at the end of 4 hours practically none of the meal had left the stomach, the experiment was discontinued. The dosage was reduced to 5 mg for all subsequent work, and the results reported here are based entirely on this dose.

According to a recent textbook of pharmacology(5), the effects of nalorphine in man, when morphine or other narcotic has not been given previously, are similar to those of morphine but less intense. The delay in gastric emptying reported here is at least as great as that produced by morphine, and possibly greater. There is some disagreement among various workers about the magnitude of the morphine effect on the stomach(6).

Summary. Five mg of nalorphine administered subcutaneously caused an average delay of over 2½ hours in the emptying time of the stomach of 7 human subjects.

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Effect of Growth Hormone and Testosterone on Induction of Cardiovascular Changes in Choline-Deficient Rats.[*] (22351)

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Severe choline deficiency may induce fatty and necrotic changes in cardiac muscle fibres, medial coronary lipoidosis and medial sclerosis of the Moenckeberg type in aortas of rats fed a high fat hypolipotropic diet(1-3). It is not known whether those changes are primarily due to choline deficiency or are secondary phenomena associated with the haemorrhagic renal cortical necrosis (HKL). Evidence in favor of the latter explanation is accumulating.

We have found that growth hormone and testosterone administered either alone or together to choline-deficient rats greatly enhances occurrence of HKL in animals which are in a borderline state of choline deficiency. Cardiovascular disease is increased in hormone-treated animals.

Methods. In a pilot experiment using 80 animals, Hall and Bieri's findings(4) were confirmed and borderline conditions for our final experiment were delineated. This re-

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[†] Research Fellow of Canadian Life Ins. Officers' Assn.

TABLE I. Composition of Diet WCA.

Casein	7
Peanut meal*	28
Soya protein†	5
Salt mixture‡	3
Sucrose vit. mixture‡	1
Sucrose	11
Starch	10
Lard	34
α-tocopherol acetate§	.015
Cod liver oil‖	.01
Corn oil§	1

* Extracted with 50%, 75% and 95% ethanol.

† Water washed "alpha proteins" (Glidden Co.).

‡ For details see: *Canad. J. Med. Sci.*, 1953, v31, 135.

§ Fat-soluble vitamins dissolved in corn oil so that 1% of dietary corn oil will supply the desired amount of fat-soluble vitamins.

‖ Obtained from Ayerst McKenna and Harrison, Montreal; contains 200,000 i.u. vit. A and 50,000 i.u. vit. D/g.

port deals only with this final experiment in which 44 female rats were used. Using our high-fat severely choline-deficient diet, it was found that weight range between 110 and 115 g in female rats was just suitable, at a room temperature of 72 degrees, for production of the desired borderline conditions in control animals. All animals were put on the same choline-deficient diet, the composition of which is shown in Table I. One experimental group was injected with 3 mg of growth hormone subcutaneously once daily; another with 1 mg testosterone (CIBA) subcutaneously once daily, while a third group was injected with both hormones. Choline-deficient animals injected with saline served as controls. The growth hormone used was obtained from the Connaught Laboratories in Toronto, lot 200, 1955 and was prepared by the method described by Campbell and Davidson(5). It contains less than 0.05 US unit TSH, less than 0.005 US unit ACTH and less than 0.2 US unit Prolactin.

The animals were injected for a maximum of 14 days. The survivors in the experimental groups were then kept another 3 weeks on a choline-deficient diet, to detect any late effect of the treatment. They were then sacrificed at the same time as the saline-injected controls.

Results. Table II illustrates the effect of growth hormone and testosterone administered either alone or together to young female choline-deficient rats. The saline-injected females all had fatty livers but the incidence of kidney and heart lesions was very low. There were no deaths from bilateral renal cortical necrosis in the control group. All control animals which showed slight involvement of the kidney due to lack of choline ("renal frosting," Hartroft(6)) were able to sustain a critical period of kidney damage between the 10th and 14th experimental day. Fatty infiltration of cardiac muscle fibres and focal cardiac necrosis were present in mild degree. The aortas of the saline-injected controls did not exhibit any lesions.

All the animals given growth hormone, however, showed a marked degree of kidney and cardiovascular involvement. The livers were fatty. More than half died from acute bilateral haemorrhagic renal cortical necrosis. The survivors in the growth hormone group had advanced degrees of 'renal frosting' as is shown in Table II. The rate and severity of cardiovascular involvement increased with the advent of HKL. At least half the aortas showed the Moenckeberg-type lesion described elsewhere(1).

The findings in the testosterone-injected animals were similar but slightly less advanced. The animals injected with both growth hormone and testosterone exhibited more severe lesions but not more frequently than the animals given growth hormone

TABLE II. Effect of Growth Hormone and Testosterone on Cardiovascular Changes in Choline Deficiency.

Group	Avg wt, g	Avg food intake, g/day	No. of rats	Pathology: Kidney: Frosting	Pathology: Kidney: HKL	Pathology: Kidney: Total	Pathology: C. V. system: Cardiac	Pathology: C. V. system: Aortic	Pathology: C. V. system: Total
Control	112	14.5	18	6	0	6	3	0	3
Growth hormone	112	14.3	7	3	4	7	4	3	7
Testosterone	110	11	9	3	6	9	6	3	6
Growth hormone + testosterone	117	12.6	10	3	7	10	5	4	5

alone. The number of animals used does not permit an accurate assessment of the effects of the different hormones. We were primarily interested in elucidating the aetiology of cardiovascular disease in choline deficiency and less so in the ability of different hormones to aggravate it.

The average daily food consumption was calculated for the first week of the experiment only. Thereafter, the experimental animals exhibited loss of weight, reduction and irregularity of food intake. The hormone-injected animals perhaps showed a slight decrease in their food intake in comparison with the saline-injected controls. (Table II).

Discussion. The observation of Hall and Bieri(4), on the aggravating effect of growth hormone on choline deficiency has been confirmed and extended. Under suitable conditions of borderline choline deficiency, growth hormone may force animals into the full-blown picture of severe bilateral renal cortical necrosis (HKL). With the development of HKL, cardiovascular changes make their appearance in increased frequency and severity. The results of our present growth hormone experiment provide further evidence(11) that the appearance of cardiac lipoidosis and necrosis, coronary medial lipoidosis and the Moenckeberg-type of arteriosclerosis in choline deficiency are probably closely associated with HKL. It is probable that growth hormone in our experiments stimulates protein synthesis(7). This would augment the methionine requirement, which in turn would increase the severity of choline deficiency, as less methionine would be available to act as a precursor of choline. The increased severity of choline deficiency may then aggravate the bilateral renal cortical necrosis and this lesion may play its part in the cardiovascular changes. This concept is supported by the fact that results similar to those obtained with growth hormone are secured by testosterone administration. Testosterone may be a powerful protein anabolic hormone(8) in the presence of insulin(9).

Growth hormone did not increase the food intake in our choline-deficient animals. It increases appetite when administered to normal animals, but this effect was not demonstrable in choline deficiency.

Selye(10) was able to produce cardiovascular changes (collagen disease like) by growth hormone and/or DCA injection in rats which were "sensitized" to these two hormones. The nature of the lesions produced does not resemble closely those which we have found. (Moenckeberg type).

Summary. 1. The work of Hall and Bieri on aggravation of choline deficiency by growth hormone has been confirmed. 2. Under suitable borderline conditions, growth hormone precipitates haemorrhagic lesions of the kidney in choline-deficient animals. With the advent of kidney lesions, cardiovascular changes appear. 3. These findings provide further evidence that the aetiology of cardiovascular lesions in choline deficiency is closely associated with kidney damage, but a causal relationship of one lesion to the other can not be established by experiments of this type. 4. Testosterone has effects similar to those of growth hormone in choline deficiency. 5. The mechanism of this aggravation of choline deficiency has been briefly discussed.

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Inhibition of Estradiol-Induced Pituitary Hypertrophy in Rats.* (22352)

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If adult rats are treated chronically with estrogens, their pituitary glands undergo changes in morphology and activity(1-4). Several natural and synthetic estrogens produce increases in weights of hypophyses and adrenals, along with other changes. Adrenal hyperplasia is apparently related to stimulation of ACTH production. This has been shown directly by bio-assay of circulating ACTH in blood of estrogen treated rats(5) and by indirect evidence. Increase in adrenal size and adrenal ascorbic acid depletion occur following estrogen treatment of intact rats(6, 7) but not in hypophysectomized rats given estrogens(8,9). Since stimulation of ACTH production and pituitary enlargement are usual consequences of prolonged estrogen treatment it seems of interest to determine if there is a causal relationship between morphologic and physiologic effects.

Synthetic estrogen tri-*p*-anisyl chloroethylene (TACE) has been reported as of no effect on pituitary size following administration of large doses for long periods of time(10). Including this compound, we compared several estrogens for their effects on pituitary weight and ACTH secretion in rats. Certain combinations were administered with total estrogenic dose adjusted so that comparisons could be made with groups receiving equi-potent doses of a single estrogen.

Methods. Dose groups of 16 male rats of Sprague-Dawley strain, weighing approximately 160 g at start of experiment, were employed. Estradiol-17β, diethylstilbestrol, and TACE were dissolved in olive oil so that each 0.1 cc contained 10 rat units. Estradiol-17β and diethylstilbestrol were administered subcutaneously. TACE was administered orally rather than subcutaneously because its mg potency is greater by this route. Estrogens were given daily for 30 days, alone and in combination in doses indicated in Table I. When combinations of estrogens were used, each was administered at a separate site. Control animals received olive oil orally and subcutaneously in volumes equal to those employed in experimental groups. Animals were sacrificed by decapitation on day following administration of last dose, and the pituitary, adrenals, thymus, and testes removed, blotted dry, and weighed on torsion balances.

Results. Estradiol or stilbestrol administration resulted in significant increase of pituitary weights, adrenal hyperplasia, and involution of the thymus glands. No pituitary hypertrophy occurred with TACE, but the effects on adrenal and thymus sizes were comparable to those obtained with other estrogens. Simultaneous administration of TACE with estradiol prevented pituitary enlargement, obtained when the same amount of estradiol is given alone. There was no significant inhibition of the estradiol-induced hypertrophy by concurrent injection of stilbestrol. However, contrary to the additive effect one might expect, a pituitary average weight resulted, lower than obtained when only estradiol is administered. The same inhibitory effect occurred when the two estrogens used in combination were stilbestrol and TACE. Combinations of 2 estrogens were neither additive nor inhibitory in regard to adrenal and thymus weight changes. Since estrogens produce adrenal hypertrophy and thymus involution only in rats with intact hypophyses, it is assumed that these estrogen effects are indirect, depending on stimulation of ACTH release from the hypophyses. Testicular atrophy, indicative of prolonged estrogenic suppression of pituitary gonadotrophin production, was approximately the same in all groups.

Discussion. That stilbestrol causes a significant, but lesser degree of pituitary hyperplasia than estradiol, and that TACE causes no increase in pituitary weight should first be considered on the basis of comparative

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TABLE I. Average Body and Organ Weights following 30 Days Treatment with Estrogens.

Groups*	Daily dose, R.U.†	Final body wt, g	Avg pituitary wt, mg	Avg adrenal wt, mg	Avg thymus wt, mg	Avg testis wt, mg
Control		230	7.4 ± .4‡	14.9 ± .6	351 ± 20	1367 ± 40
TACE	10	190	7.3 ± .4	23.2 ± 1.2	257 ± 14	645 ± 70
	20	183	7.7 ± .1	23.8 ± 1.7	232 ± 21	784 ± 95
Stilbestrol	10	203	9.1 ± .3	28.2 ± 1.0	291 ± 19	684 ± 62
	20	198	9.1 ± .4	27.1 ± 1.2	237 ± 11	730 ± 71
Estradiol	10	203	15.6 ± 1.1	28.0 ± 1.1	247 ± 18	805 ± 79
	20	209	20.6 ± 1.2	26.9 ± 1.7	225 ± 14	841 ± 74
Estradiol + TACE	10 20 10	185	9.9 ± .6	29.2 ± 4.3	264 ± 23	642 ± 53
Estradiol + Stilbestrol	10 20 10	198	12.5 ± 1.3	34.0 ± 1.1	215 ± 15	627 ± 55
Stilbestrol + TACE	10 20 10	196	8.2 ± .8	23.3 ± 1.2	254 ± 15	788 ± 76

* 16 animals/dose group.

† 10 R.U. = Estradiol, 3.2 μg; stilbestrol, 30 μg; TACE, 210 μg.

‡ ± stand. error.

doses of each estrogen used. In the design of our experiment equi-potent doses of each estrogen were approximated using a biologic criterion of activity (the rat unit), *i.e.*, the dose necessary to cause vaginal cornification within 72 hours in 50% of a group of castrated female rats. However, even this endpoint cannot serve to equate the doses precisely since duration of effect for each estrogen differs. Estradiol and stilbestrol could be termed short-acting estrogens, since vaginal cornification following a single dose rapidly disappears. On the other hand, TACE is long-acting, maintaining vaginal cornification for several days following a single administration. In this 30-day study, a difference of this nature would tend to "favor" the short-acting estrogens because the 30 daily injections would create a series of sharp peaks and drops in circulating estrogen level, whereas a more constant estrogen stimulus to the pituitary follows similar treatment with TACE. It cannot be supposed that the constancy of estrogen levels, *per se*, is the variable factor responsible for pituitary-sparing effect, since parabiosis experiments show that endogenous estradiol stimulation from enlarged ovaries of a parabiont united to a castrate partner brings about substantial pituitary enlargement(11).

Pituitary changes following estrogen combination treatments warrant special consideration. In every case, the average pituitary weight following administration of 2 estrogens fell between the averages obtained when each substance was given individually. The most striking inhibition, therefore, occurred when TACE, which causes no pituitary enlargement, was administered concurrently with estradiol, which creates the largest pituitaries. These results suggest a competitive action between the 2 estrogens, rather than an additive one as occurs when the dose of estradiol is doubled.

In addition to pituitary hypertrophy during estradiol or stilbestrol treatment, greater ACTH activity is indicated by adrenal weight increase and thymus involution. But TACE treated animals, with normal sized pituitaries, also appear to undergo some measure of adrenal stimulation. This observation suggests that estrogen-induced pituitary hyperplasia and increase of ACTH output are not necessarily related phenomena. Substantiating this view are the data showing that even though it was possible to inhibit pituitary weight increases by combining TACE with another estrogen, no inhibition of adrenal stimulation occurred in this situation.

Final consideration should be given to the question of non-specific stress caused by estrogen treatment. When estrogens are administered to rats, starvation is a possible evocative agent for adrenal changes(12), since lowered food intake is a characteristic occurrence. Thus, adrenal effects, symptomatic of increased ACTH output may result from estrogen stimulus directly, or may be caused indirectly by hunger situation created. In the present experiment, interpretation of results does not hinge on this matter since the problem under consideration is the relationship between estrogen-induced pituitary hypertrophy and ACTH output, regardless of whether the latter is directly or indirectly stimulated. Furthermore, major comparisons of adrenal effects are made among groups which have all been treated with estrogens so that food deprivation factor is constant. Food consumption values are approximately the same for each group. However, this factor bears on the problem of expressing data as average absolute gland weights or gland weights per unit body weight, for the final body weights of experimental groups were less than controls. In our results, the relationships described are in the same direction on either basis but since group differences are somewhat altered by different treatment of data a choice between the two may be pertinent. It is our belief that the data expressed as average gland weight express more precisely the actual trend of events since body weight loss of estrogen treated groups, particularly the TACE groups, is due mainly to loss of body fat. It presents a somewhat artificial analysis to adjust glandular weights according to depletion of body fat stores.

Summary. Equi-potent doses of estradiol caused greater pituitary enlargement in rats than stilbestrol. A third estrogen, TACE, caused no enlargement whatsoever. When two estrogens were given concurrently there was a competitive, rather than an additive, action. Hence, with each combination the average pituitary weight was between averages obtained when each substance was administered individually. With all estrogens there was evidence for ACTH-stimulated adrenal hyperactivity, determined by increase in adrenal weight and thymus gland involution. The observations that (1) TACE causes adrenal stimulation without pituitary enlargement and (2) combinations of estrogens can bring about an inhibition of the usual pituitary enlargement without a corresponding inhibition of adrenal stimulation suggest that the estrogen-induced hyper-production of ACTH does not depend upon pituitary hypertrophy.

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Serum Lactic Dehydrogenase Levels in Various Disease States.* (22353)

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The elevation of serum lactic dehydrogenase (LDH) activity has been observed in association with a variety of diseases. Such elevations have been reported in humans with certain neoplastic diseases(1,2) and in mice with spontaneous, induced and transplanted tumors(3,4). High LDH activity has also been observed in patients with several non-neoplastic disease states, such as diabetic acidosis, myocardial infarction and hepatitis(2), although the number of cases is small and comparative levels of activity between various non-neoplastic and neoplastic diseases have not been specified. It is evident from such preliminary observations that a broad survey of a large variety of disease states is required in order to evaluate properly the clinical significance of changes in serum LDH levels and the usefulness of such determinations as a diagnostic tool. Furthermore, such a survey might suggest possible mechanism for alterations in serum LDH levels. In this report, then, we are presenting the results of such a survey, consisting of 130 individuals free of evidence of clinical disease, and 338 unselected hospital admissions.

Material and method. Clotted venous samples from human subjects were used for this investigation. The blood was allowed to stand at room temperature for no longer than 30 minutes and then centrifuged to separate serum from blood cells. For LDH determination serum samples thus collected were diluted prior to analysis. The micromethod of Strominger and Lowry(5) was used for the determination of level of enzyme activity and the results were recorded as millimoles of substrate oxidized per liter of serum per hour. Storing of uncentrifuged blood samples at 4°C not over one day gave the same result as that obtained with samples kept at room temperature for 30 minutes or less. However, clotted blood samples which remained at room temperature for as long as 60 minutes showed an increase in LDH activity by approximately 25% over those stored at 4°C or at room temperature for no more than 30 minutes. Heparinized and oxalated blood samples also gave LDH values about 40 and 60% higher, respectively, than refrigerated or 30 minute samples. The latter were therefore excluded from the data herein presented. The data have been grouped by organ systems, with subclassification into disease groups, and, except for a graphic presentation of findings in myocardial infarction, are all recorded in Table I. Multiple determinations were made in many cases, but except for those with myocardial infarction, an average was taken in each instance.

Results. (a) Normal controls. LDH was present in all human sera tested. Levels of serum LDH activity were determined in 68 normal females and 62 normal males ranging in age from 10 to 89 years. There was no significant variation with age or sex. The average for all 130 subjects was 15.1 ± 3.01, with a range of variation between 7.5 and 22.7.

(b) Cardiovascular system. There were 87 subjects with various cardiovascular diseases in this survey, and 40 of these were individuals with acute myocardial infarctions. The number of patients whose sera were tested at each time-period following onset of clinical symptoms is recorded at the appropriate points in Fig. 1. In all instances there was a confirming electrocardiographic diagnosis. Some patients were studied for as long as one month after onset of symptoms. The highest serum LDH levels occurred during the first 3 days, with values frequently 3-4 times normal; following this there was a gradual diminution to normal, which was usually observed between the 5th and 6th days after onset. In some instances there was a secondary rise between the 15th and 20th days, which was interpreted as possibly a secondary

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TABLE I. Serum Lactic Dehydrogenase Levels in Normal and Disease States.

Group	No. of cases	Avg	Range of variation
(a) Normal			
Females	68	14.6	7.6– 20.3
Males	62	15.7	9.1– 22.7
(b) Cardiovascular			
Myocardial infarct.*	40		
Hypertensive cardiovascular disease	38	17.5	3.8– 30.8
Mitral stenosis	3	18.0	16.7– 18.8
Subacute bacterial endocarditis	1	27.8	
Pulmonary infarct.	1	22.2	
Gangrene of lower extremity	4	17.7	10.6– 23.0
(c) Central nervous			
Cerebrovascular accident†	18	17.5	8.1– 31.0
Brain tumor	2	18.9	17.8– 19.9
Psychoneurosis	6	14.1	8.4– 16.6
(d) Gastro-intestinal			
Acute infection	13	16.1	10.5– 25.0
Gastric & duodenal ulcer	13	17.4	10.4– 25.9
Carcinoma, pretherapy	8	17.7	13.2– 25.7
post-therapy‡	12	17.1	12.6– 25.3
(e) Urinary			
Acute infection	17	17.0	11.5– 24.0
Chronic nephritis	6	20.8	12.7– 27.5
Carcinoma of kidney	3	18.4	12.5– 22.1
(f) Respiratory			
Acute infection—Lung	10	18.1	10.1– 26.2
—ENT	3	15.1	9.1– 24.3
Chronic " —Lung	3	21.1	17.2– 25.7
—ENT	3	15.9	11.0– 19.9
Carcinoma of lung			
Pretherapy	3	16.1	13.4– 21.4
Post-therapy	4	18.1	13.6– 22.1
Carcinoma of larynx			
Pretherapy	5	20.3	19.8– 21.8
Post-therapy	2	17.2	15.6– 18.7
(g) ♀ genital			
Non-malignant disease	8	13.7	4.8– 18.6
Carcinoma of cervix	4	14.6	9.7– 17.6
(h) Hemopoietic			
Infectious mononucleosis	5	27.2	23.6– 30.2
Thrombocytopenia, post-splenectomy	1	37.1	
Granulocytopenia§	2	14.3	9.4– 17.6
Leukemia, stem cell and myelogenous	4	38.9	24.3– 61.5
Leukemia, chronic lymphocytic	1	13.1	
Lymphoma, pretherapy	3	23.7	21.4– 27.4
post-therapy	3	24.1	13.9– 34.8
(i) Liver, pancreas and biliary tract			
Acute cholecystitis	2	17.1	13.4– 20.7
" infectious hepatitis	1	34.2	
" cholangitis‖	2	35.2	30.2– 40.5
Thorazine hepatitis	1	103.8	
Cirrhosis of liver	7	17.8	14.7– 23.6
Chronic cholecystitis	3	20.2	12.5– 24.4
Carcinoma—metastatic to liver	4	43.2	17.6–106.8
Carcinoma, gallbladder¶	1	40.2	
Ampulla Vater‖	1	36.1	
pancreas**	4	22.8	13.9– 39.4
(j) Breast			
Benign disease	4	15.0	11.3– 18.7
Carcinoma, pretherapy	4	13.8	3.5– 23.3
post-therapy††	8	30.1	5.9–106.8
(k) Miscellaneous			
Rheumatoid arthritis	4	25.1	20.5– 27.8
Fractures	3	15.6	12.0– 19.8
Osteomyelitis	1	19.0	
Diabetes	10	15.4	7.6– 21.4
Myxedema	2	19.7	17.2– 22.2
Hyperthyroidism	1	20.6	
Minor surgery	26	15.6	6.2– 21.4

* See Fig. 1.

† Approximately 40% showed 21.4 to 31.0.

‡ 4 cases had metastatic disease.

§ One case post-splenectomy.

‖ These cases all had obstructive jaundice.

¶ No obstructive jaundice; no metastasis to liver.

** Only 1 case had liver involvement and obstructive jaundice.

†† 5 cases had levels >25, and only 1 had known massive involvement of liver.

attack, but electrocardiographs failed to substantiate such an interpretation. (In addition, one case each of pulmonary infarction and subacute bacterial endocarditis showed slight elevations in serum LDH, but these were possibly associated with small foci of myocardial necrosis.) In 38 cases of hypertensive cardiovascular disease and in 3 with rheumatic heart disease and mitral stenosis values were within normal limits. There were a few cases in the former group with values between 25 and 30, and these may have been associated with small subclinical myocardial infarcts. Surprisingly, 4 subjects with arterial thrombosis and gangrene of the lower extremity also showed LDH values within normal limits.

(c) Nervous system. Neither cerebral infarct, hemorrhage nor primary brain tumor showed average levels of LDH significantly deviating from normal. However, in about 40% of individuals with cerebral infarction and/or hemorrhage LDH levels ranged between 21.4 and 30.

(d) Gastrointestinal tract. It is also evident from the data in this category that acute infectious processes (appendicitis and di-

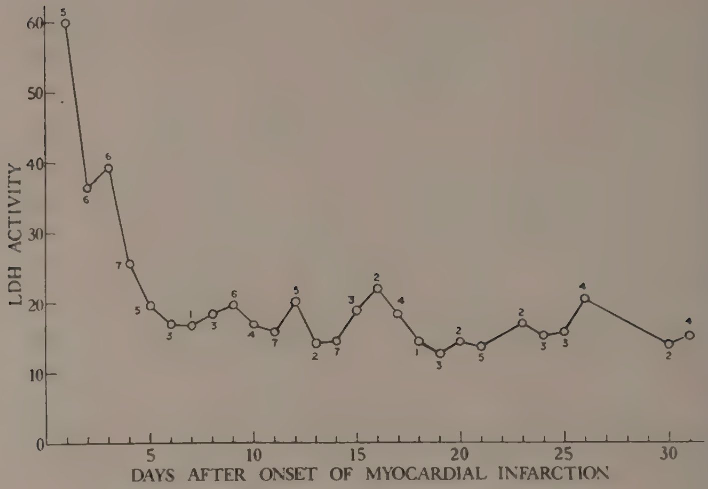

FIG. 1. Serum LDH activity in myocardial infarction.

verticulosis), gastric and duodenal ulcers, some of which were actively bleeding, and carcinoma (stomach, colon and rectum) are not associated with an increase in serum LDH activity. Furthermore, with regard to carcinoma there was no significant difference in LDH activity between patients with the primary tumor, some of whom showed widespread metastases, and those in whom the lesion had been recently resected and in whom no evidence of residual disease was present.

(e) Urinary tract. In 17 patients with acute renal and bladder infections, for the most part secondary to hypertrophy of the prostate, average LDH values were within normal limits, and this was also true of 6 chronic nephritics. There were 3 cases in the latter group with values in excess of 20, but these patients did not show elevated blood urea nitrogen levels, and in several other patients with an elevated blood urea nitrogen LDH values were also within normal limits. Urinary retention and uremia are therefore apparently not associated with elevated serum enzyme levels. In 3 instances of carcinoma of the kidney LDH values were not significantly elevated.

(f) Respiratory system. The results in patients in this group were essentially similar to those with diseases of the gastrointestinal tract. In cases with acute inflammatory disease these were for the most part instances of bronchopneumonia and acute sinusitis, and in the chronic inflammatory group chronic bronchitis and chronic tonsillitis. As regards carcinoma, there was again no significant difference between the pretherapy and post-therapy groups, the latter including patients who had undergone definitive surgery or who were receiving radiation therapy for metastatic disease.

(g) Female genital tract. In this category were included non-malignant diseases of the ovaries, tubes, uterus or cervix (benign tumors and chronic inflammation), as well as 4 cases of epidermoid carcinoma of the cervix. All showed levels of serum LDH activity within the normal range.

(h) Hemopoietic system. In the data shown under the hemopoietic system, infectious mononucleosis consistently gave moderately elevated LDH levels. LDH levels were most strikingly elevated in 4 patients, 2 with stem cell and 2 with myelogenous leukemia, but one patient with chronic lymphatic leukemia showed a normal LDH level. Patients with malignant lymphoma, including Hodgkin's Disease, showed levels comparable to that seen in infectious mononucleosis, with the exception of one case receiving x-ray therapy in whom LDH activity was within normal limits on several determinations. One patient with splenectomy for thrombocytopenia purpura showed a level about twice average normal; that this was probably not due to splenectomy as such is indicated by the fact that a post-splenectomy patient with granulocytopenia failed to show an increase in LDH activity.

(i) Liver, gallbladder, pancreas and biliary tract. As regards the 25 patients in this category it is evident that cirrhosis of the liver and inflammatory disease of the gallbladder did not produce significant change in LDH levels. However, with lesions that produce obstructive jaundice, such as common duct calculi, Thorazine and malignant tumors, as well as acute infectious hepatitis, LDH levels were elevated to about twice the normal average. Markedly elevated levels were observed in a patient with thorazine hepatitis and one with massive infarction of the liver associated with metastatic carcinoma from the breast.

(j) Breast. In the preoperative state patients with benign lesions and with malignant lesions of the breast without clinical evidence of metastasis showed no significant elevation in serum LDH. However, in 5 of the 8 cases with metastatic disease there was a significant elevation in enzyme activity, and in only one was there massive liver involvement as indicated above.

(k) Miscellaneous. In this category, only the group of 4 arthritic patients gave results deviating from normal. While these were only moderately elevated, they were, however, consistently over 20. One of these was a patient with disseminated lupus.

Discussion. Of the neoplastic diseases investigated, only stem cell and granulocytic leukemia, lymphoma and those involving hepatic metastasis were associated with a consistent elevation of serum LDH activity.

Of the non-neoplastic diseases studied, myocardial infarction, infectious mononucleosis, thrombocytopenia, hepatitis, and obstructive jaundice whether intra or extrahepatic, also gave similar increases in LDH. Since lymphoid, hepatic and cardiac tissues are relatively rich sources of LDH(6), it has been commonly suggested that if necrosis occurs, the enzyme might be released from the damaged tissue into the blood stream, therefore increasing serum LDH. If the tissues involved in disease processes have a low LDH content, one would not expect any significant increase in the total LDH activity. Therefore, they should fall somewhat in the normal range. Furthermore, some neoplastic transformation may depend upon other organs involved as metastatic sites; here, bone appears to be a common metastatic site, since most of these cases showed x-ray evidence of osseous metastasis. It remains for future studies to determine whether tumors of bone, primary or metastatic, are associated with elevated LDH levels.

In the cases of leukemia and infectious mononucleosis and thrombocytopenia the release of the enzyme might be the result of two different causes. First, it could be due to a change in cellular permeability, which possibly results in the secretion of the enzyme from the cells. Secondly, since white blood cells are relatively more fragile in the case of leukemia than normal white cells, the chance of break-down of white cells must be greater and the end result would therefore be the release of enzyme into the blood stream. Other tissues involving neoplasm would be disintegrated more frequently than the normal original tissues, therefore a high normal range level of serum LDH activity was observed in these patients. Other cases with acute infection or leucocytosis of moderate or severe degree showed slight elevation of serum LDH. This elevation might be related to the causes similar to those postulated previously.

The fact that carcinoma of intestine, stomach and kidney, 3 relatively rich sources of LDH(6), gave normal level serum LDH seems to reject the suggestion that serum LDH elevation was due to the release of the enzyme from tissues. While this concept is only plausible, it could be explained as due to some inactivation processes. The enzyme in the gastrointestinal and urinary tracts might be rendered inactive before it entered the blood stream. Furthermore, studies on the rate of urinary excretion of LDH in various neoplastic and non-neoplastic diseases may also prove fruitful.

Skeletal muscle and brain tissue of mouse were also pointed out as comparatively rich sources of LDH(6); however, there is no comparable study in human beings. If this finding could be applied to man, then gangrene of the lower extremity should give more striking results than myocardial infarction, and cerebro-vascular accidents should give data comparable to the latter. However, a normal level of serum LDH was noted. This might be explained as follows: The enzyme released from the necrotic tissues in the myocardium have ready access to the flowing blood stream in the cardiac chambers, thus resulting in an elevated serum LDH. Whereas in the leg and brain when occlusion of the major channel of blood occurs, the released enzyme does not have ready access to a blood supply, so the enzyme is destroyed or inactivated before reaching the circulatory system.

Our present studies did not confirm the elevation of serum LDH levels in diabetic acidosis as reported by Wróblewski and LaDue (2). Two of the 10 diabetic patients in the present investigation were in acidosis and the highest value obtained was 21.4. This might be due to the difference of degree in diabetic acidosis.

The mechanism by which serum LDH is increased in association with certain diseases is yet unknown. Experiments relating to the etiology may lead to a clearer understanding of this phenomenon. At present serum LDH determination may be used as a supplementary tool for diagnosing certain neoplastic diseases. It is a good index for myocardial infarction and diseases involving hepatic change.

Summary. The serum lactic dehydrogenase activity of normal human subjects and of patients with various diseases has been studied. Elevated levels were found in such neoplasms as stem cell and granulocytic leukemias, lymphomas, carcinoma of the pancreas and gallbladder, metastasis from carcinoma of the breast, and in metastatic disease of the liver. Increased enzyme activity was also noted in such non-neoplastic diseases as myocardial infarction, infectious mononucleosis, thrombocytopenia, acute infectious hepatitis and obstructive jaundice. Possible mechanisms of such elevation in serum LDH level have been discussed.

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Methemoglobinemia Induced by X-Irradiation.* (22354)

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As a first approximation it may be assumed that X-irradiation produces ion-pairs in aqueous solution as in air and for the same dose that the number of ion-pairs produced per unit weight is the same in solution as in air. A one-roentgen dose of X rays, by definition, produces 2.08 x 10^9 ion-pairs per 0.001293 g of air and may be considered to produce 1.6 x 10^{12} ion-pairs per ml of aqueous solution. Also, in aqueous solution containing oxygen there is reason to assume production of oxidant equivalent to 4 univalent atoms per ion-pair. Thus, one roentgen produces the equivalent of 6.4 x 10^{12} univalent atoms of oxi-

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* Supported by U. S. Atomic Energy Commission.

dant per ml of solution. Since one microequivalent corresponds to approximately 6 x 10^{17} univalent atoms, a one-roentgen dose of X rays will produce the order of 10^{-5} microequivalents of oxidant per ml of solution. This estimate of the oxidizing ability of X rays is based on the simplest assumptions about mechanism and probably is somewhat low(1); however, the observed degree of oxidation of most biological materials is less than expected from the estimate(2). A recent report(3) indicates that 25,000 roentgens of X rays applied to lysed human erythrocytes diluted 1:40 with phosphate buffer converted about 60% of the hemoglobin to methemoglobin. For present purposes erythrocytes may be considered to contain 20 microequivalents of ferrous iron in the form of hemoglobin. The solution of hemoglobin irradiated contained, therefore, 0.5 microequivalents of hemoglobin iron per ml. The dose of irradiation used would provide 0.25 microequivalents of oxidant per ml and might, therefore, convert 50% of the hemoglobin to methemoglobin. The conversion reported, 60%, although large compared to results obtained with other biological material(2), is about the same as might be expected from the oxidizing potential of the radiation. The report(3) also indicates that irradiation of the rat *in vivo* with 3000 roentgens led to the conversion of about 2% of the hemoglobin to methemoglobin. This represents oxidation of 0.4 microequivalents of hemoglobin per ml of erythrocytes. A second dose of 3000 roentgens given immediately after the first led to the oxidation of additional hemoglobin. The amount oxidized by the second dose was about twice that obtained with the first dose. 3000 roentgens would provide 0.03 microequivalents of oxidant per ml of erythrocytes and might, therefore, convert 0.15% of the hemoglobin to methemoglobin. Thus, the amount of hemoglobin found oxidized was 13-26 times as much as expected.

It is possible that practically all of the oxidizing potential of the radiation arising throughout the animal appeared in the erythrocytes by way of the circulation. Assuming the animals had a total volume of 300 ml, a red-cell volume of 15 ml, and all of the oxidant arising from 3000 roentgens eventually appeared in the red cells, each ml of the cells would be subjected to 0.6 microequivalent of oxidant. This amount is of the same order as the amounts of hemoglobin found oxidized by 3000 roentgens. An alternative explanation is that the effect of the irradiation *in vivo* was multiplied in some fashion. The relatively small chemical potential of a dose of radiation that will profoundly affect an animal, *e.g.* 1000 roentgens, has given rise to speculation about the possibility of a multiplication factor, *e.g.* chain reactions. No biological system considered *in vitro* has shown the degree of multiplication implied by the experiments under consideration(2).

Experimental. The preceding considerations appear in order although in the present work using another strain of rat given up to 5000 roentgens of whole-body X-irradiation (250 KVP, 0.5-2.8 mm Cu HVL) essentially no methemoglobin was detected in the blood. Test for methemoglobin was made as follows: Immediately after irradiation the animal was anesthetized with ether. Blood was taken from the heart in heparin. An equal volume of cold water was added. The mixture was kept in the cold for about 15 minutes. An equal volume of cold saturated ammonium sulfate, adjusted to pH 6.6 with fixed alkali, was added. After standing 15-20 minutes in the cold, the mixture was filtered. The clear filtrate was assayed spectrophotometrically for methemoglobin(4). In addition, the filtrate was tested for change in optical density at 630 mμ following addition of cyanide(5) and was examined with a hand spectroscope for the characteristic absorption band of methemoglobin.

In both irradiated and control rats the level of methemoglobin did not exceed 0.2% and no difference between the groups was observed.

Summary. Methemoglobinemia was not observed in rats given up to 5000 roentgens of whole-body X-irradiation *in vivo*.

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Influence of Vit. E Deficiency in Rabbit on Urinary Excretion of Free Amino Acids.* (22355)

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It has been shown that urinary excretion of free amino acids is elevated in patients with progressive muscular dystrophy(1). Also it has been reported that vit. E-deficient rabbits excrete extra quantities of free amino acids in urine although no actual data have been presented(2,3). In view of these considerations, and since any information concerning metabolic disturbances in vit. E deficiency may aid in elucidation of the biochemical role of the vitamin, we have determined the influence of vit. E deficiency on urinary excretion of amino acids by rabbits.

Methods. White New Zealand rabbits, weighing initially about 500 g each, were given the same purified diet previously described(4) except that folic acid was omitted. Controls received this diet with oral supplements of 10 mg of alpha tocopherol acetate 2 times per week. There were 4 rabbits in each group. The animals were kept in individual metabolism cages and daily urine samples were collected throughout the experiment. Urine samples were analyzed for free amino acids by the copper method(5). The experiment was terminated when unsupplemented animals exhibited early stages of muscular dystrophy.

Results. Vit. E deficiency led to a considerable increase in excretion of free amino acids in urine. At the time of appearance of earliest physical signs of muscular dystrophy urinary excretion of free amino acids was tripled (Table I). Actually there was a significant increase in excretion of amino acids before any physical signs of dystrophy were apparent. This agrees with the observation of Ames (2).

TABLE I. Influence of Vit. E on Average Daily Urinary Excretion of Free Amino Acids by Rabbits.

	mg amino N/kilo body wt	
Days on diet	Without vit. E	With vit. E
1- 5	6.6	5.2
6-11	5.9	4.5
11-15	4.5	4.3
16-20	5.7	3.7
21-25	8.6	3.3
26-31	13.5	4.5

Increased excretion of free amino acids in vit. E deficiency may indicate an impaired incorporation of amino acids into protein, an increased breakdown of tissue proteins, or an impairment in normal metabolic disposal of amino acids. Experiments with C^{14} labeled glycine have indicated that vit. E deficiency does not significantly affect incorporation of that amino acid into protein or its oxidation to CO_2(6). This would suggest that elevated excretion of free amino acids in vit. E deficiency is due to accelerated breakdown of tissue protein, probably primarily skeletal muscle.

Summary. Vit. E deficiency in the rabbit results in increased excretion of free amino acids in urine. This increase in amino acid excretion is apparent before any physical signs of dystrophy are observed.

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Sedimentation Behavior of Desoxypentose Nucleic Acids from Normal and Pathologic Human Spleens. (22356)

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Previous work in this laboratory has demonstrated that the desoxynucleic acids (DNA) of normal and leukemic human cells differ in some physico-chemical characteristics(1,2). Interest in this study of macromolecular structures of normal and malignant cells has been greatly stimulated by the "template hypothesis"(3-5). According to this hypothesis, neoplastic growth would be the consequence of an abnormal event in the self duplication of some macromolecules in the cell(6), resulting in a malignant transformation of some cytoplasmic and/or nuclear structures.

Although the "template hypothesis" concerns primarily the role of ribonucleic acid (RNA) in the mechanism of specific protein synthesis, it could be extended to the role of DNA in mitosis and gene duplication. For this reason the sedimentation constant of DNA from normal and leukemic cells was studied.

Material and methods. DNA was prepared from calf thymus, normal human spleen removed surgically after traumatic rupture, human spleens from cases of multiple myeloma, Brill-Simmers disease, myeloid leukemia and hereditary spherocytosis. The organs were frozen with dry ice immediately after removal and powdered in a high speed mixer with additional dry ice. Thirty grams of powdered tissue were suspended in 100 ml of NaCl-Na Citrate solution (0.14 M NaCl; 0.01 M Na Citrate) and homogenized for 2 minutes at 12,000 r.p.m. The material was then filtered through 3 layers of gauze, centrifuged, and the sediment washed 3 times. The sediment was then resuspended in 20 volumes of 1.7 M NaCl-0.01 M Na Citrate solution and homogenized again for 30 seconds at a lower speed. DNA sodium salt was extracted according to the method of Gulland (7) as modified by Chargaff *et al.*(8), then dialyzed against distilled water for 72 hours at 0-4°C and lyophilized. The N/P ratio as well as other chemical properties of the obtained DNA, correspond closely to the theoretical values and have been published elsewhere(2). DNA was dissolved in cacodylate buffer of ionic strength 0.3 and pH 7(9) by gentle overnight stirring in the cold room (0-4°C). The DNA concentration of the solution was calculated from its phosphorus content (P DNA) determined with the method of Fiske and Subbarow, after conversion of the arsenic from the penta- to the trivalent form(10). The samples were analyzed at room temperature within 24 hours of being dissolved. Sedimentation data were obtained in the Spinco Model E analytical ultracentrifuge. Sedimentation constants were corrected for a solvent having viscosity and density of water at 20°C; the partial specific volume of DNA was assumed to be 0.55(11,12).

Results. Typical schlieren photographs of DNA sedimentation boundaries are seen in Fig. 1. The data obtained with DNA from a series of normal and pathological spleens are shown in Fig. 2 where the S_{w20} values are plotted against the P DNA concentration of the samples. It can be seen that points fall on the same curve, regardless of the source of the DNA samples.

Discussion. The spread of values obtained with any given P DNA concentration seems to indicate a variability within each determination rather than differences in the sedimentation constant of DNA samples of dif-

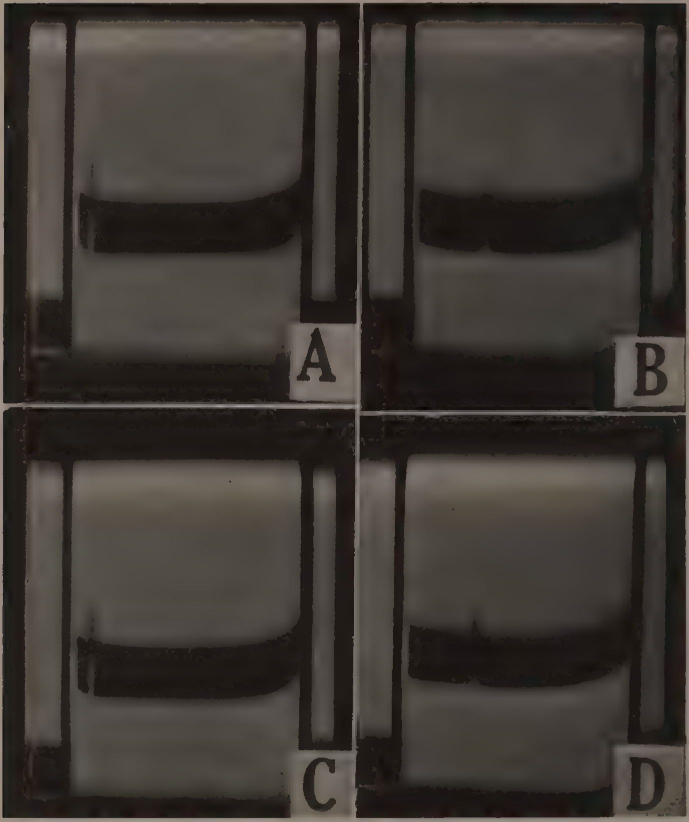

FIG. 1. A and B. Schlieren photographs of sedimenting boundaries of DNA from spleens of patients with multiple myeloma. Speed, 59,780 rpm. Bar angle, 45°. Time, 16 min. Concentration, 1.7 mg P DNA/100 ml. C and D. Schlieren photographs of sedimenting boundaries of DNA from spleens of patients with myeloid leukemia. Speed, 59,780 rpm. Bar angle, 45°. Time, 16 min. Concentration, 1.9 mg P DNA/100 ml.

ferent origin. These results do not support the contention that DNA from normal and pathological human cells have different sedimentation constants. Extrapolation of the data to concentration zero indicates an S_{w20} value of about 20 to 21, in agreement with the data of Peacocke and Schachman(9) and of Doty(12). Other authors(12-16) have reported lower S_{w20} values at zero concentration although at a concentration of DNA of 9-18 mg %, the sedimentation coefficients reported by them are in agreement with ours. This difference in the extrapolated values may be due to the different concentration range of the samples and to the different ionic strength of the buffer solutions used by various investigators. Sadron(17) and Jordan (18) have shown that some physical properties of DNA, such as viscosity, are strongly influenced by the ionic concentration of the solution, and that reproducible data can be obtained only when all free negative charges of DNA are saturated. Furthermore Peacocke and Schachman(9) have demonstrated that, when DNA is sedimented in distilled water at a concentration below 60 mg %, it behaves like degraded DNA and that the sedimentation constant increases up to an ionic strength of 0.3. Further addition of electrolytes has no greater effect.

Very strong intermolecular forces act between DNA molecules at relatively high con-

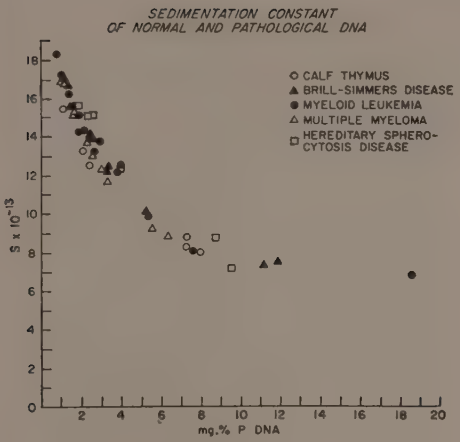

FIG. 2.

centrations, and may disturb the sedimentation rate even at the lowest concentrations used in this work. This may make the demonstration of heterogeneity in various DNA samples difficult. While this suggests the need for further studies of the sedimentation behavior of DNA at low concentrations with more sensitive recording systems (such as the optical absorption method used by Butler and Shooter(19)), the results reported in this paper indicate that DNA samples from normal and tumoral cells give identical sedimentation curves over the large range of concentrations studied.

Summary. 1. Sedimentation curves of DNA from spleens of normal individuals and patients with diseases causing a leukocyte infiltration in the spleen were obtained by means of the ultracentrifuge. 2. DNA was dissolved in cacodylate buffer (pH 7; u 0.3) at concentrations range 1-20 mg % of P DNA. No differences in sedimentation curves of DNA from normal and infiltrated spleens were noted.

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Comparative Study of Vitamin B_{12} Assay in Urine. (22357)

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In a study of cyanocobalamin (vit. B_{12}) excretion in the urine of patients, following an intramuscular (I.M.) dose of 50 μg of crystalline B_{12}, striking variations in the apparent content of B_{12} were observed(1,2). These variations stemmed from differences in the specificity of the assay organisms. It is known that some of the microorganisms serving for the microbiological assay of B_{12} respond to congeners of the vitamin and also deoxyribonucleosides(3,4). Specificity, better than that of bacteria, is shown by *Euglena gracilis* and, above all, by *Ochromonas malhamensis* (5,6). Here we compare the response of *Lactobacillus leichmannii, E. gracilis* and *O. malhamensis* to the B_{12} family of vitamins as they occur in urine.

Methods. The maintenance media for the 3 organisms have been given(7-9). The assay technics used have been outlined in previous publications(10). *L. leichmannii* grew in 24 hours, the protozoans within 4-6 days. The inoculum for the bacillus was prepared by 1:10 dilution with basal medium of a 24 hour culture. The inoculum for *E. gracilis* was prepared by 1:50 dilution of a 4-6 day culture in the basal medium. The *Z* strain rather than the *bacillaris* strain of *Euglena* was employed, as it attained higher densities in a shorter period and had the same sensitivity and specificity(11,12). *O. malhamensis* inoculation was directly from a 4-6 day culture in the maintenance medium without significant carry-over of B_{12} activity. The assay media for the organisms were given(6, 8,13). Urines from control patients, who had not received I.M. B_{12}, were diluted 10 X with buffer at pH 4.5,[†] and autoclaved for 30 minutes to convert bound cobalamin into its microbiologically active form. This procedure allows estimation of total B_{12}(8). All urines were diluted, as necessary, for the measurements to fall on the straight-line portion of the standard curve. Urines from control patients were further diluted 1:2 - 1:100 with assay medium (final dilution of the urine 1:20 - 1:100). Urines from patients, who received 50 μg of B_{12} I.M., were diluted 50 X, autoclaved, and subsequently diluted again 1:2 - 1:100 (final dilution 1:100-1:5000). *L. leichmannii* was incubated at 37° for 24 hours; the protozoans were kept at room temperature (not exceeding 30°C) for 4-6 days under constant illumination provided by six 40 W. warm white fluorescent tubes at an average distance of 0.8 m. Growth was measured as optical density units with a Welsh Densichron equipped with a red-sensitive probe.

Results. Recovery of added vit. B_{12}: To test the validity of the assay methods, recovery experiments of B_{12} in urine were done with 4 concentrations of B_{12} (Table I). The 2 protozoans yielded satisfactory recoveries from urine. In recovery experiments with sera and cerebrospinal fluids, we noticed that *O. malhamensis* may be slightly stimulated by other materials in cerebrospinal fluid and serum when compared to *Euglena*. These stimulants did not affect the results in urine.

B_{12} in Urine. Table II shows results with urine collected during the first 8 hours after intramuscular injection of 50 μg of vit. B_{12} in 23 individuals. In all except 2 urines (Nos. 1 and 5) values obtained with *O. malhamensis* were lower than for that with *Euglena*. The values with *L. leichmannii* were higher than that with *Euglena* except in 2 cases (Nos. 18

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[†] One-half gram of trans-aconitic acid/100 ml of distilled water, to which triethanolamine had been added to reach pH 4.5. One hundred mg of Na metabisulfite had also been added to 100 ml of buffer for stabilization of the analogs of B_{12} e.g. hydroxycobalamin(14,15).

TABLE I. Recovery of B_{12} Added to Normal Undiluted Urine.

Conc. (μg/ml)	*L. leichmannii*	*E. gracilis*	*O. malhamensis*
5	10.8	3.6	3.7
10	16.5	8.4	9.5
15	24.1	14.2	14.2
20	31.0	18.2	18.2

and 19). In no instance were values with *L. leichmannii* lower than with *O. malhamensis.*

Discussion. Ford(6) has evaluated the response of the 3 microorganisms to pseudovitamin B_{12} and to other B_{12}-like factors. This evaluation is used here as the basis for calculation of true B_{12} values as compared with values for pseudo-B_{12}'s. Ford and Hutner also have discussed the positive response of *L. leichmannii* to deoxyribosides(4). The main difference of practical interest between *Euglena* and *Ochromonas* is the high response of the former to pseudo-vitamin B_{12}. With *L. leichmannii* the response to pseudo-B_{12} is half that of *Euglena*. We assume that the response to *O. malhamensis* (OM) gives the true value for vit. B_{12}. Therefore: $B_{12} = OM$. The extra response of *Euglena* (E) as compared with *Ochromonas* is assumed to reflect the pseudo-B_{12}'s (Ps_{12}) in the sample. Pseudo-B_{12}'s may be estimated by the formula $Ps_{12} = \frac{E - OM}{8}$.

Besides responding to certain pseudo-B_{12}'s, *L. leichmannii* (L) also responds to thymidine and certain other deoxyribosides(3). The quantity of these deoxyribosides (D) may be computed in terms of true vit. B_{12} by the following calculation

$$D = L - OM - 4\frac{E - OM}{8}$$

which leads to $D = L - \frac{OM + E}{2}$.

These formulae are only crude approximations, as they disregard the possible presence of other members of the vit. B_{12} group. If by these calculations interference by deoxyribosides is suspected, it might be desirable to assay for them directly(16). Results in Table II indicate a correlation between pseudo vit. B_{12} and true B_{12}. Deoxyribonucleosides, and other factors, stimulating the growth of *L. leichmannii* appear to interfere in the vit. B_{12} load tests described here; the difference in results between *L. leichmannii* and the others are possibly due to dietary differences of patients, which may be reflected in their urine.

The recoveries of true B_{12}, according to Table I, appear to be 6-11 μg too high with *L. leichmannii*. This excess may perhaps be ascribed to the combination of stimulatory urinary constituents with materials present in the basal medium(17), a reaction, which assumes disturbing proportions, when the growth is dense.

Summary. The response of *Ochromonas malhamensis, Euglena gracilis,* and *Lactobacillus leichmannii* to vit. B_{12} in normal urine and in urine of patients, given an intramuscular dose of 50 μg of vit. B_{12}, was studied. The "true" vit. B_{12} values presumably are given by the *Ochromonas malhamensis* assay. The response of *E. gracilis* and *L. leichmannii* to other members of the B_{12} group and to other metabolites is discussed from the standpoint of how to estimate responses other than *O. malhamensis*-active B_{12}. Calculations are given for the estimation of interfering metabolites.

TABLE II. B_{12} in Urine of Patients within 8 Hours after Intramuscular Injection of 50 μg of B_{12}.

Total output in μg determined by response of *Ochromonas*	*Euglena*	*Lactobacillus*
.6	.5	1.2
1.0	2.4	3.0
1.0	2.9	10.6
1.5	1.7	5.0
1.9	.7	3.8
2.5	4.2	8.3
3.6	5.6	9.9
3.8	7.4	7.4
4.1	6.9	8.5
4.9	10.2	23.4
6.0	14.5	33.0
6.3	11.6	16.8
6.6	13.1	20.0
8.1	10.2	20.4
9.0	9.5	19.0
10.2	16.1	19.5
10.5	22.7	33.1
11.4	38.0	24.7
11.7	17.1	11.5
11.9	15.0	16.1
13.7	23.6	33.0
13.8	24.7	27.6
28.7	30.2	31.3

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Molybdenum Toxicity in the Rat.* (22358)

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The symptoms of molybdenum toxicity have been thoroughly described in several species(1), yet little is known of the underlying defects responsible for these symptoms, except that the resultant anemia can be remedied by copper sulfate supplementation. Weil-Malherbe and Green(2) and Lutwak and Sacks(3) have noted that compounds such as adenosine triphosphate (ATP) and acetyl phosphate are rapidly hydrolyzed in the presence of molybdenum salts. Since molybdate ions *in vitro* catalyze the acid hydrolysis of "high-energy" phosphate esters, the possibility existed that molybdenum might have a similar effect *in vivo*. If this were so, a decrease in the supply of "high-energy" phosphate compounds might then be fundamental to some of the symptoms of molybdenum toxicity. The acetylation of p-aminobenzoic acid (p-ABA) and the conjugation of glycine with benzoic acid have been used extensively in tests for liver function. Both reactions involve ATP, coenzyme A, magnesium ions, and the appropriate enzymes. Lipmann(4) has shown that ATP is involved in the acetylation of sulfonamides by pigeon liver preparations, and Chantrenne(5) has established a requirement for ATP in the enzymatic formation of hippuric acid. If molybdate ions *in vivo* should catalyze the hydrolysis of organic phosphate esters, then the supply of "high-energy" phosphate compounds might be reduced, and presumably, the reduction would be reflected in the ability of the animal to acetylate p-aminobenzoic acid or to conjugate benzoic acid.

The experiments reported here tested this hypothesis. Studies were also conducted on the effect of molybdenum toxicity on the alkaline phosphatase activity of various tissues.

Methods. The basal diet had the following

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percentage composition: casein, 20.0; glucose, 69.0; corn oil, 6.6; salt mixture, U.S.P. XIV, 4.0; choline chloride, 0.1; 2500 I.U. vit. A, 250 I.U. vit. D, 15 mg alpha-tocopherol in corn oil, 0.3. Other vitamins were provided in the following quantities, mg/100 g diet: thiamine hydrochloride, 0.5; riboflavin, 0.8; niacin, 4.0; pyridoxine hydrochloride, 0.5; calcium pantothenate, 4.0; biotin, 0.04; folic acid, 0.2; menadione, 0.5; vit. B_{12}, 0.003. The basal diet was supplemented with 4.0 mg copper/100 g in order to provide sufficient copper to prevent anemia. Albino rats of the Wistar strain were obtained from commercial sources and housed in individual cages. In the first experiment sodium molybdate was added to provide a dietary level of 0.08% molybdenum. The average initial weight of the 8 pairs of rats studied was 94 g. For each pair, the food intake of the control rat fed the basal diet was limited to the intake of the rat fed the molybdenum-supplemented diet. After one week on the experimental diets, the rats were placed in metabolism cages and injected intraperitoneally with a 2.5 mg dose of p-aminobenzoic acid. Urine was collected for 24 hours after injection. This procedure was carried out once a week for 4 successive weeks with each pair of rats. The animals were sacrificed at the end of 6 weeks. In the second experiment, the dietary levels of molybdenum were 0.08%, 0.12% and 0.14%. The average initial weight of the rats was 79 g. All diets were fed *ad libitum*. After 2, 3, and 4 weeks, rats from each group were injected with a 2.5 mg dose of p-aminobenzoic acid as described in Exp. 1. Total and free urinary p-aminobenzoic acid were measured by the method of Bratton and Marshall(6). Urinary hippuric acid was measured following injection of benzoic acid by the method of Alexander *et al.*(7). Alkaline phosphatase activity was determined by the method of Bessey *et al.*(8), in which the hydrolysis of p-nitrophenylphosphate to p-nitrophenol is measured. Activities are expressed as micromoles of phosphate released in 30 minutes per mg protein. Tissues were homogenized in appropriate amounts of ice water in a Ten Broeck homogenizer. The protein content of the homogenates was estimated by the method of Lowry *et al.*(9).

TABLE I. Effects of Dietary Molybdenum in Pair-Fed Rats.

Group	Body wt (g)	Urinary acetyl-p-aminobenzoic acid (% of total p-ABA excreted)	Liver alkaline phosphatase (%)
Control	191	49.0 (28–72)	100
+Mo 800 p.p.m.	188	53.6 (37–80)	177

Eight pairs of rats used. Urinary p-ABA values represent means of 4 replicates at 1 wk intervals or 32 assays. Range of values is given in parentheses. Liver alkaline phosphatase values are means of 8 assays.

Results. Growth and food intake. In the first experiment (Table I), the addition of molybdenum to the diets at a level of 0.08% caused a negligible depression in growth in contrast to previous results from this laboratory when younger animals from a different commercial source were used(10). Since a dietary level of 0.08% molybdenum failed to produce a severe toxicity in the first experiment, 3 levels of molybdenum were used in Experiment 2 and, in addition, younger animals were used. In this experiment, molybdenum, at all 3 levels, definitely retarded growth. The average weights at 5 weeks were 171, 121, and 87 g, respectively, for the rats fed the 0.08%, 0.12%, and 0.14% levels whereas the average weight of the control group was 218 g (Table II). Many of the rats developed an intermittent diarrhea, but there was no mortality. Prior to start of the experiment, all the rats gained normally on the basal diet. However, within 2 days after the start of the experimental period, the presence of molybdenum in the diet depressed food intake, especially at the two higher levels, and the weight gains reflected the reduced food intake. This was not due to decreased "palatability" of the diet since 5 rats given sodium molybdate by stomach tube, also showed poor food intake.

Acetylation of p-aminobenzoic acid. The addition of 0.08% molybdenum to the diet in the first experiment did not alter the ability of the pair-fed rats to acetylate a 2.5 mg dose of p-aminobenzoic acid (Table I). Likewise,

TABLE II. Influence of Various Levels of Dietary Molybdenum on Rat Growth, Tissue Phosphatase, and Acetylation of p-ABA.

Groups	Body wt (g)	Tissue alkaline phosphatase Liver	Heart	Kidney	Urinary acetyl p-ABA (% total p-ABA excreted)
		μM phosphate/30′/mg protein			
Control	218	.041 ± .004	.224 ± .021	3.709 ± .102	67 (47–87)
+Mo 800 p.p.m.	171	.085 ± .008	.178 ± .013	3.671 ± .192	53 (46–60)
+Mo 1200 p.p.m.	121	.263 ± .025	.203 ± .027	2.395 ± .364	60 (44–83)
+Mo 1400 p.p.m.	87	.220 ± .016	.218 ± .006	2.477 ± .166	52 (42–68)

Urinary p-ABA values were obtained on 6 rats/group; values in parentheses give range of values.

higher levels of molybdenum had little effect on the acetylation (Table II). Under these conditions, then, the supply of "high-energy" phosphate esters, and presumably the amount of acetyl coenzyme A available for the acetylation of p-aminobenzoic acid, was not affected by the presence of molybdenum *in vivo*.

Conjugation of benzoic acid. Following the injection of benzoic acid, the hippuric acid was isolated from urine in a one part butanol, 5 parts chloroform mixture, hydrolyzed and the glycine determined as formaldehyde with chromotropic acid. A considerable variation in response was observed to the intraperitoneal injection of 24 mg of benzoic acid. Both control and molybdenum-toxic animals were inconsistent in the excretion of hippuric acid, however, there did not appear to be any tendency for the toxic animals to excrete less of the conjugate. In one experiment of 10 control and 10 toxic rats the mean excretion was 2.2 and 2.8 mg glycine of hippuric acid, respectively, in 24 hours following administration. This supports the observations on p-ABA acetylation that molybdenum does not interfere to any extent in the occurrence of these endothermic reactions. It seems reasonable that molybdenum does not have an effect because of the higher pH of the tissue systems or due to a conjugation of the mineral with proteins of the tissues.

Tissue alkaline phosphatase. While the gross effects of molybdenum were rather slight in the first experiment, in every case, liver alkaline phosphatase activity was greater in the molybdenum-fed animal than in the pair-fed control (Table I). Although the significance of the increase in enzyme activity is unknown, the activity of this enzyme does seem to be a sensitive indicator of dietary molybdenum, and may be of potential use in the detection of molybdenum toxicity.

In the second experiment (Table II) all 3 levels of molybdenum significantly increased the activity of the liver enzyme ($P<0.01$), and the activity was significantly greater in the rats fed the 0.12% and the 0.14% levels than in the rats fed the 0.08% level ($P<0.01$). Increasing the level of molybdenum from 0.12% to 0.14% produced no additional increase in liver alkaline phosphatase activity. Heart alkaline phosphatase was unaffected.

Upon autopsy, the kidneys of the rats fed the 0.12% and the 0.14% levels of molybdenum were noticeably paler than those of the control rats or the rats fed 0.08% molybdenum, although the kidneys of the latter animals frequently were paler than those of the controls. These differences appeared consistently although the gross structure of the kidney seemed normal. This alteration, together with the possible role of alkaline phosphatase in the reabsorption of glucose in the kidney tubule(12), suggested that a study of kidney alkaline phosphatase might be valuable. The data in Table II show the effect of molybdenum on kidney alkaline phosphatase. In the rats which had been fed the diets for 4-7 weeks, the 0.12% and 0.14% levels of molybdenum significantly ($P<0.02$) depressed the activity of the kidney enzyme.

Some studies were made of the influence of dietary molybdenum on intestinal alkaline phosphatase activity. It was observed that after one week on the experimental regimen 5 rats receiving 1200 p.p.m. molybdenum had significantly less ($P<0.01$) intestinal phosphatase activity than 5 controls. Control and toxic values were 16.58 ± 1.64 and 4.46 ± 0.73 μM phosphate released/30′/mg protein respectively. Because of this rapid and pronounced effect, the influence of sodium molybdate on the intestinal phosphatase assay system was tried. Molybdate concentrations up to 10^{-4} M had no influence on a semi-purified preparation of intestinal phosphatase. At 10^{-3} M molybdate, a 20% inhibition of activity was observed. This is in agreement with experiments on liver preparations(10) and suggests that the decrease in activity observed in toxicity is probably a reflection of altered synthesis of the enzyme rather than a direct influence of the mineral on the enzyme assay system.

Summary. 1. High levels of dietary molybdenum did not alter ability of rat to acetylate p-aminobenzoic acid or to conjugate benzoic acid with glycine. 2. A depression of food intake and growth was evident within 24 hours after addition of 0.12% molybdenum to the diet. 3. Liver alkaline phosphatase activity was significantly increased in molybdenum-toxic rats whereas the activities of the kidney and intestinal alkaline phosphatases were significantly reduced.

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Structure and Function of Mitochondria in Irreversible Hemorrhagic Shock, I.*† (22359)

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Irreversible hemorrhagic shock is seen in humans and experimental animals who have suffered a prolonged period of hemorrhagic hypotension and are refractory to blood volume replacement therapy. Battlefield experience with massive blood transfusions in World War II and the Korean conflict(1) strengthened the belief of some workers that an irreversible intracellular metabolic change was taking place. Several studies involving tissue analyses have appeared during the last decade and have been ably summarized by Engel(2). In general, high levels of glucose lactate, pyruvate, amino acids and inorganic

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phosphate were found in the blood while the tissue stores of glycogen, ATP[‡], and coenzymes appeared depleted. Edwards *et al.*(3) recently repeated some of the key measurements in dogs. Early enzymatic studies were conducted by Russell, Long and Wilhelmi(4, 5) Burdette(6) and Hannon and Cook(7) using liver and heart slices of rats subjected to hemorrhagic shock: in some of these experiments lowered oxygen uptakes were observed. Thus, both the analytical and the enzymatic data suggested failure at the mitochondrial level. Harman(8-11) described a series of degenerative changes occurring in mitochondria when isolated by differential centrifugation and exposed to various environmental conditions. These alterations in structure (swelling and "crescent" formation) were accompanied by functional changes *e.g.* low P/O ratios and variations in oxygen uptakes.

The two lines of thought, *i.e.* the evidence suggesting mitochondrial involvement in the phenomenon of irreversibility in clinical hemorrhagic shock and the interesting correlation between mitochondrial structure and function observed in *in vitro* experiments raised the following questions: Are mitochondria morphologically and functionally altered in irreversible hemorrhagic shock and do such *in vivo* changes resemble the *in vitro* changes described?

Methods. Production of shock.[§] Healthy mongrel dogs maintained on a standard *ad libitum* diet of dried dog biscuits were paired as nearly as possible as to sex, age, breed and weight and anaesthetized with 30 mg/kg pentobarbital sodium. One animal served as a control while the other was subjected to hemorrhagic shock by a modification of the Wigger's technic(12). Blood was withdrawn rapidly so that an arterial pressure of 40 mm Hg was produced in approximately 5 minutes. Subsequently blood was withdrawn and reinfused as necessary to maintain the initial hypotensive level.[‖] The animals were sacrificed after 3 to 5 hours by removing the left ventricular musculature surgically. The members of the pair died within 15 minutes of each other. *Formalin-fixed preparations.* 5 g of heart muscle cut into thin slices were transferred to a semi-micro Monel cup containing 1 g sodium acetate in 50 ml of cold 10% formaldehyde and were ground on a Waring blendor base at full speed for 45 seconds. Liver and kidney samples were prepared in the same manner but blendorized at half speed for 15 seconds. The homogenates were strained through cheese cloth and examined under oil immersion in a Spencer phase contrast microscope using Dark-M contrast.

Isolation of mitochondria. 15 g heart were minced and ground for 90 seconds in 75 ml semi-frozen 0.38 M sucrose containing 100 μM K_2HPO_4 using a semi-micro Monel cup in a standard Waring blendor base. Another 75 ml of the sucrose solution were stirred in and the suspension strained through cheese cloth. The preparations from the control and the shocked dogs were thus made within 15 minutes of each other and centrifuged simultaneously in a refrigerated international centrifuge. The mitochondria were sedimented between the speeds of 450 and 1300 x g, suspended in 0.5 M sucrose, dispersed by means of a glass homogenizer and the densities of the suspensions adjusted nephelometrically. The total nitrogen concentration was estimated by Johnson's method(13). The mitochondria were thus isolated in hypertonic sucrose because of Harman's findings(10,11) that under such conditions degenerative changes ("crescent" formation) were negligible, an observation which we have been able to confirm. *Enzymatic activities* were estimated by the conventional Warburg method at limiting levels of mitochondria under the conditions outlined in Fig. 4. In order to determine P/O ratios the incubation mixtures were fortified with 50 μM fructose and 2 mg

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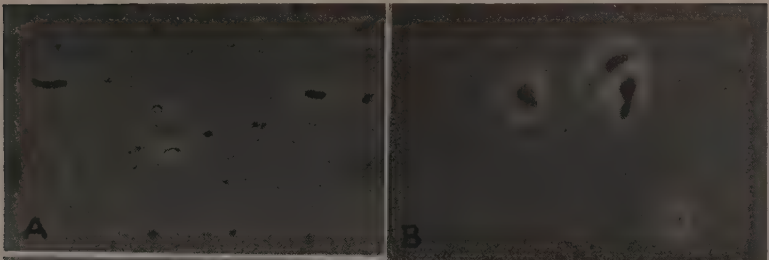
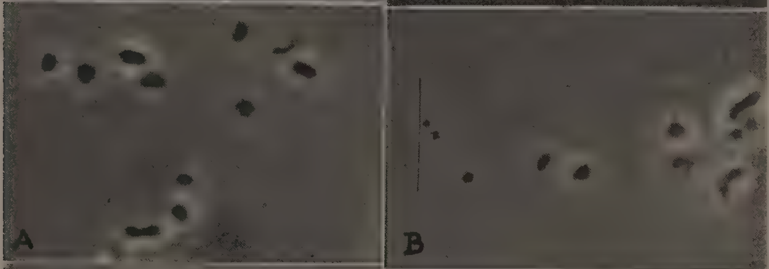
[‡] The following abbreviations will be used: AMP = adenosine − 5′ − phosphate; ATP = adenosinetriphosphate; P/O ratios = micromoles of inorganic phosphate esterified per microatom of oxygen consumed.

[§] The experiments described were carried out between July and Nov. 1955.

Baxter blood bottle with ACD solution 1.38% Na-citrate, 1.58% dextrose and 0.5% citric acid were used to collect the blood.





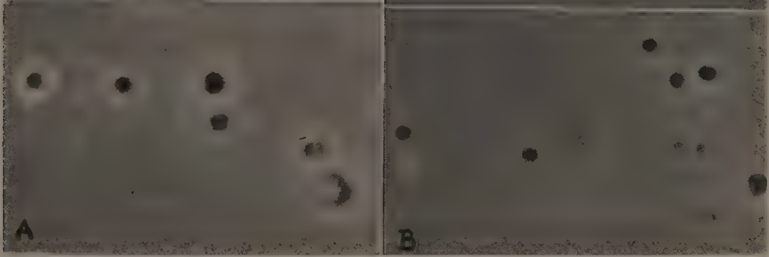


FIG. 1 (top). Heart mitochondria from normal and shocked dogs prepared in formaldehyde and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

FIG. 2 (middle). Heart mitochondria from normal and shocked dogs isolated by differential centrifugation, suspended in 0.5 M sucrose and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

FIG. 3 (bottom). Heart mitochondria from normal and shocked dogs aged in 0.5 M sucrose and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

yeast hexokinase (Sigma, type II) which were poured in from the side arm shortly before the taps were closed. Inorganic phosphate levels were determined by a modification of the Fiske-Subbarow method. In a few experiments fluoride was added as a phosphatase inhibitor but the results were not affected materially by its presence. In general the experimental mixtures were kept as simple as possible in an effort to maintain the mitochondria in as nearly their native state as feasible.

Results. Morphology of formalin-fixed preparations may be observed in Fig. 1. The grinding breaks up the long chains of mitochondria which accompany the strands of myofibrils in the undisturbed tissue. The free floating normal particles tend to be square to spindle-shaped rods with sharp boundaries and dense to moderately translucent centers. At times they may be slightly knobby. The particles encountered in preparations from shocked dog hearts are swollen and irregular in shape with indistinct boundaries and often have translucent centers. Blendorates from livers and kidneys also showed abnormal mitochondria. The swollen spheres and "crescents" which are associated with degenerative changes in Harman's *in vitro* experiments have not been observed in formalin-fixed preparations.

Structure of mitochondria isolated in su-

TABLE I. Oxygen and Phosphate Uptakes of Heart Mitochondria from Normal and Shocked Dogs in the Presence of the Hexokinase System.

Substrate	Normal			Shock			
	O_2 uptake µatoms /mg N*	P uptake µmoles /mg N*	P/O	O_2 uptake µatoms /mg N*	P uptake µmoles /mg N*	P/O	Hr of shock
α-Ketoglutarate	12.1	19.5	1.6	10.3	11.8	1.1	2
	9.9	16.9	1.7	10.4	20.7	2.0	3
	9.8	25.9	2.6	5.9	13.8	2.3	3½
	11.4	23.4	2.1	5.1	8.3	1.6	5
	9.8	22.7	2.3	6.8	10.5	1.5	5
	8.6	14.8	1.7	4.9	3.6	.7	5
	14.8	30.9	2.1	8.8	14.8	1.7	5½
Succinate	10.4	19.6	1.9	11.7	13.2	1.1	3
	14.3	31.3	2.2	13.5	20.6	1.5	3
	10.8	22.5	2.1	11.0	23.0	2.1	5

* Avg of duplicate or triplicate flasks.

Main compartment: 20 µM $MgCl_2$, 3 µM AMP, 50 µM phosphate buffer, pH 7.5, 40 µM substrate, mitochondria (app. 1 mg N) and 0.5 M sucrose to 1.8 ml; sidearm: 50 µM fructose and 2 mg hexokinase in 0.2 ml; center well: 0.2 ml 2 N NaOH; temperature: 30°C; gas phase: oxygen.

crose suspension is demonstrated in Fig. 2. Rods persist in varying numbers, often as high as 80%; the remaining mitochondria appear as spheres of varying sizes. "Crescents" are not seen in fresh suspensions. Preparations from shocked dogs generally have a lower rod count and show a preponderance of the small and medium sized spheres while the normal tends to contain large forms. This difference in size is often very marked and is observed also in the incubation mixtures at the end of Warburg experiments. A comparison of Fig. 1 and 2 reveals that the normal mitochondrion tends to be small in formalin but large in sucrose whereas the abnormal behaves in an opposite manner.

On aging at room temperature swollen spheres and "crescents" will be formed in both types of preparations. However, this *in vitro* change tends to occur less readily and less extensively in the particles isolated from shocked dogs (Fig. 3). *Enzymatic data.* Fig. 4 shows that the oxidation of succinate, malate and α-ketoglutarate was essentially unimpaired even after 5 hours of shock; in many experiments the 2 curves were superimposable. Experiments with acetate and butyrate were also negative. No requirements for coenzyme I, guanosine diphosphate¶ or cytochrome c could be demonstrated; in fact, added coenzymes often were found to be inhibitory. On the other hand, Table I indicates that in the presence of the hexokinase system oxygen uptake and phosphate esterification were often decreased: thus in seven experiments with α-ketoglutarate the normal consumed an average of 11 microatoms of oxygen per mg nitrogen and 22 micromoles of phosphate resulting in a P/O of 2.0; this was reduced in shock to 7.5 microatoms oxygen and 12 micromoles phosphate

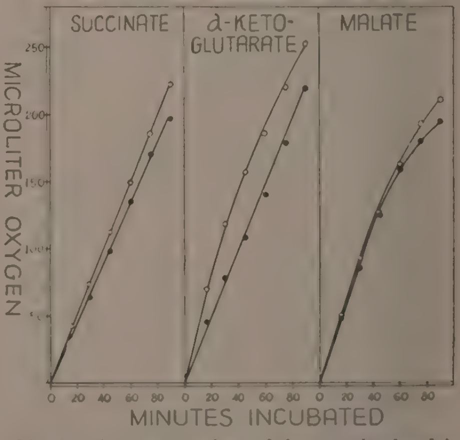


FIG. 4. Oxygen uptakes of heart mitochondria from normal and shocked dogs. Main compartment: 20 µM $MgCl_2$, 3 µM ATP, 50 µM phosphate buffer, pH 7.5, 40 µM substrate, mitochondria (app. 1 mg N) and 0.5 M sucrose to 2.0 ml; center well: 0.2 ml 2 N NaOH; temperature: 30°C; gas phase: oxygen. ○ = normal, ● = 5 hr shock.

¶ Generously presented to us by Dr. D. M. Gibson of the Enzyme Institute.

giving an average P/O of 1.6.

Discussion. It may be concluded, therefore, that the mitochondria are structurally altered in hemorrhagic shock and that these *in vivo* changes differ from the *in vitro* degenerations described by Harman, for in the formalin-fixed preparations the basic rod shape is maintained despite the swelling and distortion.

Upon isolation in sucrose solution, however, the serial transformations observed by Harman are found to take place, *i.e.* the rods change into spheres which increase in size and ultimately form "crescents." Under these conditions the spheres derived from the abnormal mitochondria are smaller and exhibit less of a tendency to swell and to turn into "crescents." This phenomenon suggests that shock alters the particles in a way which renders them less responsive to environmental conditions. It will be of interest to study their submicroscopic internal structure.

The observation that the *in vitro* oxidative activities of the abnormal mitochondria are affected by the presence of the hexokinase system has raised an interesting point. It is possible that the classical Warburg methods, which supply an optimal environment as far as cofactors and substrate requirements are concerned, obscure or reverse early enzymatic defects. The negative results of Fig. 4 could thus be explained. In the living organism, on the other hand, the mitochondria are called upon to furnish an undiminishing supply of energy and building materials for use by other cell constituents. It may well be that the hexokinase trap which sets up a constant extramitochondrial demand for ATP mimics to some extent such *in vivo* situations. The results of the enzymatic studies reported here would then indicate that the heart mitochondria of shocked dogs are capable of generating enough energy to maintain themselves in a functional state but begin to fail when exposed to stress conditions which force them to produce ATP at rates beyond those which will suffice for their maintenance. Future work will have to elucidate the correctness of this hypothesis.

Summary. Dogs were subjected to irreversible hemorrhagic shock. Their hearts, livers, and kidneys contained mitochondria which differed in size and shape from those of normal dogs. Isolated by differential centrifugation the mitochondria from shocked animals were found to retain the normal oxidative capacities for Krebs cycle substrates but tended to have impaired activities in the presence of the hexokinase system.

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Some Factors Influencing Potentiation of Cardiac Contractility Following an Extrasystolic Beat.* (22360)

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The increase in contractility of cardiac muscle following an extrasystole was first described by McWilliams(1). Cattell and Gold (2,3) have confirmed and extended this finding and Garb and Penna(4) have determined the effects of the interval between the basal and extrasystolic beats on the potentiated contraction in cat papillary muscle. The present report deals with some of the factors influencing post-extrasystolic potentiation (PEP) of cardiac contractility as determined in the isolated rabbit auricle.

Methods. The isolated quiescent rabbit auricle suspended in Tyrode solution was used. Details of this preparation and the Tyrode solution used are found in a publication by Rosin and Farah(5). The bath temperature was controlled at 37.0-37.5°C. Electrical stimulation of the auricle was accomplished by means of 2 coupled Grass stimulators (Model S4C). The first stimulator was used for driving at basal rates and served to trigger the second stimulator which provided the extrasystolic beats. The interval between basal and extrasystolic beat as well as voltage and pulse duration could be varied at will. Extrasystolic stimuli were interjected through the same electrodes which delivered the basal stimulus or at a point 2 to 4 mm from the latter electrodes. Results in both instances were essentially the same. Contractility was recorded by means of a Grass force transducer and a Grass inkwriting electroencephalograph. Quinidine sulfate, when used, was added to the bath in a dosage of 0.25-1.0 mg% and readings were made 30 minutes after addition of the drug.

Results. Post-extrasystolic potentiation of cardiac contractility could be demonstrated both in the isolated auricle and isolated ventricular strips of the rabbit. The quantitative data to be reported have been obtained on the quiescent isolated rabbit auricle stimulated at basal rates of 0.2 to 5 beats per second.

Fig. 1A illustrates a typical result. It can be seen that a single extrasystolic beat produced a potentiation of contractility which was increased by interjecting a second and a third extrasystolic beat. This cumulative effect of extrasystolic beats has been regularly observed and usually reached a maximum following 5 to 8 such stimulations. Maximum potentiation of cardiac contractility thus attained exceeded 100 and sometimes attained 300 to 400%. The contraction velocity of the post-extrasystolic beat is accelerated as the peak of the contraction occurs only slightly later than that of the pre-extrasystolic beat (Fig. 1B).

It is difficult to state whether the electrical extrasystole which produced potentiation also produced a mechanical extrasystole. In many of our experiments (see Fig. 1A) there were no readily visible mechanical extrasystoles. However, close inspection of some of the tension curves frequently showed a slight irregularity in the descending part of this curve which may indicate a mechanical change. With the recording methods used it is impossible to decide whether an electrical or both electrical and mechanical extrasystole were essential for the production of potentiation. It is hoped to resolve this problem and such studies are now being initiated.

The effect of resting tension on the production of PEP was studied. In general PEP was observed with low or high initial tensions. Usually an increase of the initial tension by 1 to 2 g increased the height of the basal beat as well as PEP. On occasion an excessive increase in tension would reduce both the basal beat and the PEP. This is in conformity with

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* Supported by a grant-in-aid from the American Heart Assn.

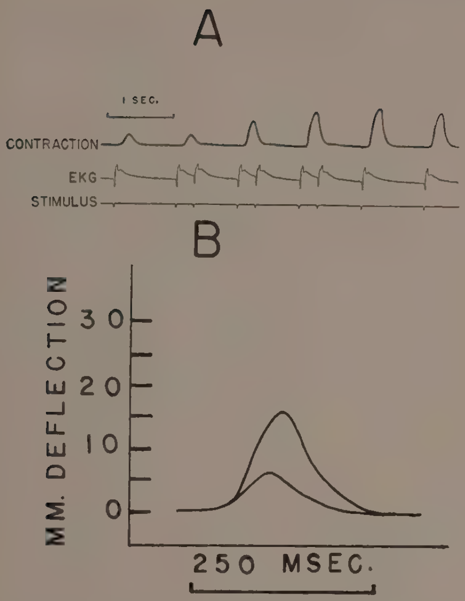


FIG. 1A. Effect of 3 extrasystoles on contractions of isolated left auricle of rabbit. Temp. 37.5°C. Tyrode solution. Note increase in contractile tension following interjection of the extrasystoles.

FIG. 1B. Superimposed records of contractions obtained from isolated left auricular preparation. Lower trace: basal contraction; upper trace: contraction following a single extrasystolic stimulus delivered 80 milliseconds after basal stimulus. Basal rate 2 per sec. Temp. 37°C.

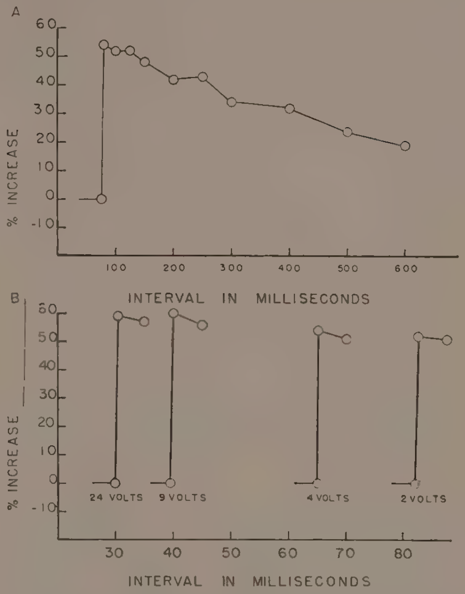


FIG. 2A. Effect of interval between basal and extrasystolic beat on post-extrasystolic potentiation in rabbit auricle. Basal rate = 1 per sec. Temp. 37°C. Height of first post-extrasystolic beat expressed as % of basal beat. Stimulus 15 volts.

FIG. 2B. Effect of stimulus intensity on PEP in rabbit auricle. Conditions as in Fig. 2A except for stimulus intensity.

Starling's law of the heart. The PEP was not accompanied by a measurably different action potential from that of the basal beat (Fig. 1A). In general, the first post-extrasystolic beat showed maximal potentiation, however, the second, third, and even fourth beats following a single extrasystolic beat would show some potentiation. The decay of potentiation followed an exponential curve and in this respect our results confirm those of Cattell and Gold(3), Garb and Penna(4), and Rosin and Farah(5).

The interval between the extrasystolic beat and the post-extrasystolic beat was varied between 1 and 120 seconds. It could be shown that PEP was still apparent after a rest period of 120 seconds. In this respect the results are qualitatively similar to those of Garb and Penna(4) and Rosin and Farah(5) who determined the effect of rest period on post-extrasystolic potentiation, and post-stimulation potentiation of cardiac muscle respectively.

The effects of the interval between basal and extrasystolic beats on PEP were studied in 25 auricles. In general the shorter the interval the greater the PEP until an interval was reached when the auricle would not respond (Fig. 2A). If a threshold stimulus was used the interval necessary to produce maximal potentiation was longer than when supramaximal stimuli were used (Fig. 2B). The shortest possible interval could be obtained by using stimuli equal to 6 to 9 times threshold and this minimal interval corresponded to the effective refractory period, as determined from a strength-interval curve. In most experiments maximal potentiation was seen when the interval between basal and extrasystolic beat was minimal. However, in some experiments maximal potentiation did not

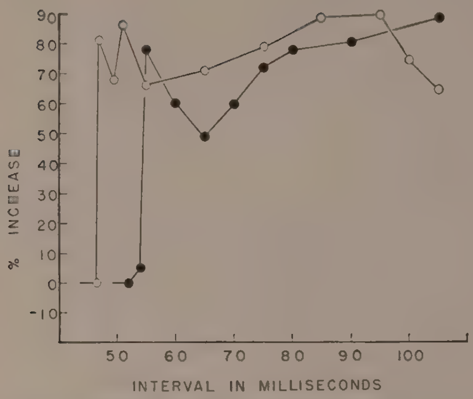


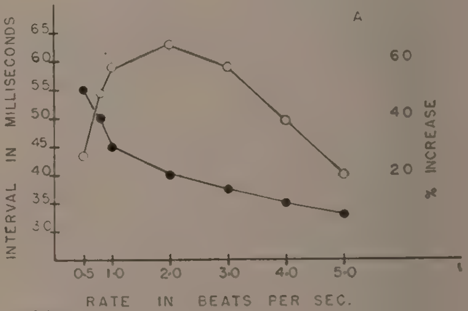
FIG. 3. Effect of interval between basal beat and extrasystole on 1st post-extrasystolic beat showing atypical response curve. Rabbit auricle. Temp. 37°C.

correspond to the effective refractory period but occurred at longer intervals (Fig. 3). We do not have any explanation for this variation, and it was observed only in a few preparations; however, these changes may correspond to the dips in the excitability curve described by Brooks, *et al.*(6).

Since rate of stimulation influences the refractory period of cardiac muscle, we have determined the effects of rate on the PEP in the isolated rabbit auricle. It could be shown that an increase in rate produced a shorter effective refractory period as determined by strength-interval curves and decreased the time interval between the basal and extrasystolic beat necessary to produce a maximal potentiation (Fig. 4A). The observation was made that an increase in rate of stimulation not only decreased the time interval for PEP but also increased the extent of potentiation (Fig. 4B) up to rates of about 3 per second; at higher rates both basal and potentiated beats were reduced. This finding is different from that of Garb and Penna(4) who did not find this relation of rate of stimulation of cat papillary muscle to the magnitude of the PEP.

At high rates of stimulation (4.0-5.0/sec.) the potentiation would occasionally be delayed, *i.e.*, no potentiation of the first post-extrasystolic beat but marked potentiation of the second would be observed. Garb and Penna(4) and Rosin and Farah(5) have also observed this delayed potentiation which occurs only at high rates of stimulation.

Quinidine is known to prolong the effective refractory period of cardiac muscle. We have thus determined the effects of quinidine on the interval necessary for the production of PEP as well as the extent of the potentiation produced. Following control readings quinidine was added in increasing cumulative concentrations to the bath and both strength-interval curves and PEP were determined. The data are given in Fig. 5 and it can be seen that quinidine in concentrations of 0.25 to 1.0 mg % prolonged the interval necessary to



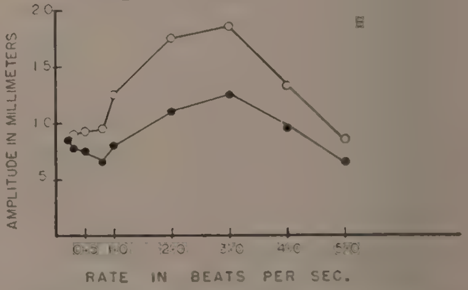


FIG. 4A. Effect of rate of basal stimulation on minimal interval between basal beat and extrasystolic which produced post-extrasystolic potentiation in rabbit auricle. Temp. 37°C. ●—● Minimal interval between basal beat and extrasystole producing potentiation of the first PEP. ○—○ % change in tension of PEP as related to basal beats at each rate.

FIG. 4B. Effect of rate of basal stimulation on post-extrasystolic potentiation. Avg of 4 auricular preparations. Temp. 37°C. Post-extrasystolic potentiation measured at minimal interval between basal and extrasystolic beat producing potentiation. Abscissa: Basal rate. Ordinate: Tension of isometric twitch expressed in millimeters of pen deflection. ●—● Basal beat. ○—○ Potentiated beat.

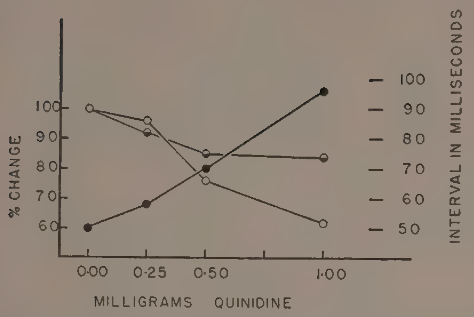


FIG. 5. Effect of quinidine on interval between basal beat and the extrasystole which produced maximal PEP in isolated rabbit auricle. Temp. 37°C. ●—● Minimal interval in milliseconds between basal beat and extrasystole which produces maximal PEP. *Half-solid circles*—Contractility at basal rate (1/sec.) expressed as % of control. ○—○ Change in PEP expressed as % of control.

produce PEP. Quinidine decreased the extent of the potentiation but also decreased the height of the basal contraction. However, the per cent decrease in the potentiated beat exceeded the changes in the basal beat (Fig. 5). It is thus likely that quinidine in some way interfered with the process or processes responsible for the initiation of post-extrasystolic potentiation.

Discussion. The presence of post-extrasystolic potentiation (PEP) of cardiac beats was demonstrated in both isolated auricular and ventricular muscle strips of the rabbit. This phenomenon of PEP has been observed by others in isolated cardiac muscle preparations (1-4). In our own experience PEP occurs in the isolated heart of dogs, rabbits and cats perfused by the Langendorff technic. Furthermore, PEP has been observed in occasional auricular and ventricular strips of the rabbit when spontaneous extrasystoles occurred. In the dog heart-lung preparation we have observed that the appearance of one or more extrasystoles would significantly improve the cardiac function especially if the heart showed signs of decompensation. The effects of such extrasystoles became apparent by a temporary decrease in the right auricular pressure and cardiac size, a temporary increase in cardiac output and stroke volume. All these changes have been frequently observed but have been explained on the basis of changes in cardiac filling and "Starling's law of the heart." The observations in the heart-lung preparation are difficult to analyze since changes in fiber length occur and thus the relation of PEP to changes in fiber length cannot be clearly formulated. Recently Siebens, *et al.*(7) studied this phenomenon in the dog heart and changes in fiber length of the ventricle could be excluded as a cause of post-extrasystolic potentiation. It is thus likely that the PEP studied in the isolated auricular and ventricular strip is not characteristic of the isolated preparation alone but also occurs in blood-perfused, intact hearts.

The relation of post-extrasystolic potentiation to post-stimulation potentiation and to the staircase (Treppe) phenomenon should be considered. In our experience the behavior of these 3 phenomena responds to physical as well as pharmacological changes in the same directions and it is likely that the underlying fundamental mechanisms for all 3 types of potentiation are the same. As regards the mechanism of post-extrasystolic potentiation little can be said at this time. Ellis(8) has suggested that glucose-phosphate is related to the staircase phenomenon. Recently Ritchie and Wilkie(9) have concluded that the staircase phenomenon and post-tetanic potentiation in frog striated muscle were caused by the delay in the fall of the active state of muscle postulated by Hill(10). It is possible that the active state of muscle is related to post-extrasystolic and post-stimulation potentiation. The active state persists for milliseconds while the potentiation of contractility persists for several beats and can be elicited minutes after the extra-systolic beat. These properties of PEP and post-stimulation potentiation make it difficult to explain these phenomena solely on the basis of a prolongation of the active state. A process which is only slowly reversible and which is dissipated by the following contractions may precede the active state and may determine the length of the active state and velocity of contraction.

The PEP of auricular muscle is related to the refractory period since an increase or decrease in the refractory period also produced parallel changes in the PEP.

Summary. Post-extrasystolic potentiation (PEP) of cardiac contractility was studied in rabbit auricular muscle. It is shown that potentiation is related to the interval between basal beat and extrasystolic beat. An increase in driving rate up to about 3 per second increased the extent of potentiation and decreased the interval between basal beat and the extrasystole necessary for the production of maximal potentiation. Quinidine increased the effective refractory period and the interval for producing PEP and decreased the degree of potentiation produced by the extrasystole.

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Metabolic Actions of Acetic Acid Analogs of Thyroxine and Triiodothyronine.* (22361)

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In the field of thyroxine analogs and possible derivatives which may have special metabolic characteristics, the most striking recent observations were those of Gross and Pitt-Rivers(1) and of Roche, Lissitzky and Michel(2) that 3,5,3′-triiodothyronine (Trit) was several times more active than thyroxine. Many workers have since confirmed this on a variety of test animals(3). The time of delay before the metabolic effects of Trit are seen is now recognized as shorter than with thyroxine(4-7), but still present. The duration of Trit action is also more brief, effects which are more striking in the myxedematous human than in experimental animals(8,9).

Thibault and Pitt-Rivers(10,11) reported that 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac) and 3,5,3′-triiodothyroacetic acid (Triac) produced an immediate *in vitro* stimulation of kidney and liver slices appearing at concentrations about 0.01 μg Triac per ml or 0.05 μg Tetrac per ml and reaching a maximum at respectively, 1.0 and 5.0 μg per ml. In addition, Tetrac was stated to cause a considerable rise in metabolic rate of thyroidectomized rats in 2 hours followed by a return to the starting level after 4 hours. Pitt-Rivers had previously reported on the activity of Tetrac and Triac(12), noting no unusual results except for unexpectedly high toxicities. The present report concerns possible qualitatively distinct properties of Tetrac and Triac.†

Methods. All rats used were from the Sprague-Dawley strain, thyroidectomized at least 6 weeks previously. Most tissues were prepared as previously discussed(13). Ehrlich mouse ascites tumor cells, cultured *in vivo* in Carworth white mice,‡ were washed sev-

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* This work was supported by grants from the Council on Pharmacy and Chemistry of American Medical Assn., the Smith, Kline, and French Foundation, and Committee on Growth of American Cancer Society.

† Samples of Tetrac and Triac were obtained through the generosity of Dr. Pitt-Rivers and of Dr. John A. King, Warner-Chilcott Laboratories.

‡ These cells from a Southern Research Institute culture were obtained through the courtesy of Dr. Warner W. Carlson and Mr. J. T. Huggins.

TABLE I. Effect of Triac or Tetrac Added *In Vitro* to Tissue Slices Prepared from Thyroidectomized Rats.

Compound added, µg/ml	No. of animals	% of control Q_{O_2}					
		Kidney		Liver		Heart	
		0–15	0–60	0–15	0–60	0–15	0–60
Triac: .01	8	99.3	100.0	102.0	103.5	115.0	111.4
.10	8	97.2	100.0	101.7	102.3	107.5	111.1
1.0	8	94.1	97.3	97.1	93.7	104.9	110.4
5.0	2	97.9	99.0				
10.0	8	95.2	96.1	93.7	89.4	85.7	84.7
Tetrac: .05	8	87.8	92.7	93.8	88.3		
.50	8	96.9	97.1	101.4	98.7	96.4	100.8
1.0	2	103.6	100.9				
5.0	8	95.7	97.8	105.5	102.7	86.7	98.3
10.0	2	98.2	95.3			77.4	91.7
50.0	8	73.4	65.1	101.0	101.8	52.7	55.7

eral times by suspension in Krebs' Ringer solution followed by mild centrifugation. A 10% v/v suspension was made from the final packed tumor cells using a solution appropriate to the determination of oxygen consumption (Krebs' Ringer-phosphate) or glycolysis (Krebs' Ringer-bicarbonate). Any thyroxine analog being studied was present in 0.005 N NaOH final concentration. Control vessels always contained the same amount of alkali and 0.9% NaCl.

Results. Findings are divided into 3 groups: (1) *In vitro* effects on rat tissues, (2) *In vivo* injection into thyroidectomized rats, (3) *In vitro* effects on tumor cells.

1. *In vitro Triac and Tetrac effects on tissue metabolism.* Table I shows oxygen consumption values calculated as percentages of control values for 0-15 and 0-60 minutes of observation. Liver, kidney and heart were not stimulated significantly over these time intervals. Kidney was clearly depressed by 50 µg Tetrac/ml solution and heart by both 10 µg Triac/ml and 50 µg Tetrac/ml. Heart also appeared to have been inhibited the first 15 minutes by 5 and 10 µg Tetrac/ml, but escaped by 1 hour. Liver metabolism was not altered markedly by either material, although it was slightly depressed by the highest level of Triac. Readings actually were continued for a second 60 minutes, with no significant changes beyond those already discussed. Rates of shaking of the apparatus were also varied over the range of 80 to 120 per minute. The data published by Thibault and Pitt-Rivers(10) for kidney yield the following averages: with 5 µg Tetrac/ml solution, 73% increase for 15 minutes and 30% for 60 minutes; with 1 µg Triac/ml, 106% and 30% for 15 and 60 minutes, respectively. In no instance did the results obtained in this laboratory approach these increases. The later report by these workers(11) indicated a more evanescent effect of Triac on liver slices. Concentrations from 0.01 to 1.0 µg/ml all gave increases averaging 28% for 15 minutes and 7% over 60 minutes.

2. *In vivo effects of injected Triac and Tetrac.* The reported immediate increase of BMR in thyroidectomized animals produced by Tetrac injection(10) might be due to undesirable non-specific effects. We have studied the metabolism of tissues removed from thyroidectomized rats 1.5 hours after injection of 2 mg Tetrac/kg or 1 mg Triac/kg. The tissues were immediately chilled to 5°C and kept at this temperature throughout the period of preparation, about 0.5 hour. The effective time after injection is considered to include only the 15 minute equilibration time at 37° in addition to the 1.5 hours after injection.

The results in Table II fail to show any rapid response following injection of the acetic acid analogs into thyroidectomized animals. Heart was included because it has been found to respond with such high sensitivity to injected thyroxine and skeletal muscle because it makes up so much of the metabolizing body mass. Preliminary experiments with liver and kidney have shown the same absence of immediate energy metabol-

TABLE II. Oxygen Consumption of Tissues Removed from Thyroidectomized Rats Injected with Tetrac (2 mg/kg) or Triac (1 mg/kg).

Hr after inj.*	% of control Q_{O_2}					
	Tetrac-injected			Triac-injected		
	Heart	Diaphr.	Skel. M.	Heart	Diaphr.	Skel. M.
1¾–2	94.9	94.4	109.2	102.8	97.8	94.7
2 –2¼	106.9	96.1	105.3	97.9	93.4	93.4
2¼–2½	96.3	90.0	104.6	98.1	102.9	90.8
2½–2¾	92.3	91.9	113.6	100.3	97.3	95.5
2¾–3¼	111.1	94.9	105.0	92.2	96.6	88.3
3¼–3¾	88.0	92.5	100.0	89.5	98.1	83.6

* Not including approximately ½ hr required for preparation of tissues, during which time tissues were kept at 0°C. Four animals used in each category.

TABLE III. Metabolism of Tissues Excised from Thyroidectomized Rats Injected with Thyroxine, Tetrac or Triac.

Tissue	Thyroid-ectomized Q_{O_2}	% increase above control Q_{O_2}		
		L-thyroxine*	Tetrac*	Triac*
Liver	.98	59.2	53.1	52.0
Diaphragm	.72	34.7	23.6	33.3
Heart	.44	170.6	147.7	277.3
Skel. M.	.68	27.9	17.6	23.5
Pancreas	.83	39.8	20.5	25.3
Saliv. gl.	1.86	31.7	22.0	20.4
Kidney	3.27	35.5	21.7	45.9

* Compound specified in each case was inj. at a dose of 2 mg/kg body wt/day for 4 days and animal sacrificed on fifth day. Three animals in each group.

ism change.

Thyroidectomized rats were also injected with Triac and Tetrac at several dose levels once per day for 4 days, and sacrificed on 5th day. The most consistent responses were obtained at a level equimolar to 2 mg thyroxine/kg/day. These are shown in Table III, together with responses to L-thyroxine.§ The cumulative effect from these materials, as with thyroxine, suggests that metabolism is stimulated for longer than 2 to 4 hours. It may be estimated that Tetrac is about three-quarters as active as L-thyroxine. Since Triac stimulated tissues to approximately the same extent as thyroxine, it is about one-fourth as active as L-triiodothyronine. The apparently greater effect of Triac on the heart requires further study.

3. *In vitro responses of Ehrlich's mouse ascites tumor cells.* This tumor cell appears to be a useful test object with Triac and Tetrac(14). Table IV gives the Q_{O_2}, $Q^{O_2}_{CO_2}$ and $Q^{N_2}_{CO_2}$ values obtained in terms of the wet weight of tumor tissue. Since the dry weight averaged 11.0% of the wet weight, multiplication by a factor of 9.1 yields dry weight Q's. The endogenous metabolic rate was the same as that with pyruvate, but glucose caused a 40% decrease. The nature of the foodstuff used in the absence of added substrate is not known, but the repeated washings with saline should have removed such substances as pyruvate or lactate. As is typical of tumor cells, addition of glucose caused a tremendous increase in glycolysis over the non-nutrient, about 12-fold for aerobic and 32-fold for anaerobic.

The tumor cell Q_{O_2} was not affected by Triac from 0.01 to 10 μg/ml, or Tetrac 0.05 to 5, except for 8-10% increases at levels of 0.5 to 1.0 μg/ml. Triac at 50 and 100 μg/ml and Tetrac at 50 showed increasing inhibition. In contrast, as shown in Fig. 1 and 2, both aerobic and anaerobic glycolyses were enhanced by 50 μg Triac/ml, the former by 52% and the latter 22%. Somewhat lesser effects were shown by 60 μg Tetrac/ml. However, the thyroxine isomer, B-5522, N-(3,5-diiodo-4-hydroxybenzoyl) -3,5-diiodotyrosine, devoid of thyroxine-like, metabolism-stimulating properties when injected into thyroidectomized animals, also gave increases in both aerobic and anaerobic glycolyses. In addition, thyroxine itself accelerated aerobic glycolysis and Trit anaerobic.

In general, these results confirm the observations of Heimberg *et al.*(14) on the *in*

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§ Sodium L-thyroxine was kindly provided by Dr. H. L. Fevold, of the Travenol division of Baxter Laboratories.

TABLE IV. Oxygen Consumption, Aerobic Glycolysis and Anaerobic Glycolysis of Ehrlich Mouse Ascites Tumor Cells.

			Triac, μg/ml (as % of control)						Tetrac, μg/ml (% of control)			
Determ.	Substrate	Control	.01	.10	1.0	10	50	100	.05	.5	5.0	50
Q_{O_2}	None	.90				90.0	33.3	10.0				
	Glucose	.56	97.0	98.0	110.1	98.2	41.1	16.1	102.0	108.1	99.0	45.5
	Pyruvate	.98				93.9	55.1	22.4				
$Q^{O_2}_{CO_2}$	None	.14										
	Glucose	1.75				104.6	152.0	87.4				
$Q^{N_2}_{O_2}$	None	.08										
	Glucose	2.59	102.5	99.5	95.8	113.5	121.6	70.2	100.0	99.3	101.5	

vitro stimulation of glycolytic activity by Tetrac and Triac. However, our findings that thyroxine and Trit have similar effects throws considerable doubt on interpreting these changes as being specifically related to an action of the iodothyroacetate derivatives. Furthermore, the comparable activity exhibited by a physiologically inactive thyroxine isomer, B-5522, suggests that the entire phenomenon is related more generally to some structural configuration of these compounds (such as the 3,5-diiodo-4-hydroxyphenyl group) than is the complete oxidative pathway. It would be quite premature to insist that one function or the other was the more "genuine" action of the thyroid hormone.

The present studies seem to put the acetic acid analogs of thyroxine and Trit into a rather less dramatic relationship to the "physiologically active" thyroid hormone than was indicated by Thibault and Pitt-Rivers. Both Triac and Tetrac are thyroactive compounds, but show no clearly qualitative differences from thyroxine or, more appropriately, Trit. Other studies being carried out at present indicate that Triac, Tetrac and Trit may all produce a more rapid response on the sensitive heart than thyroxine when injected into thyroidectomized animals. Whether the acetic acid analogs have, also like Trit, a more rapid turnover, remains to be determined.

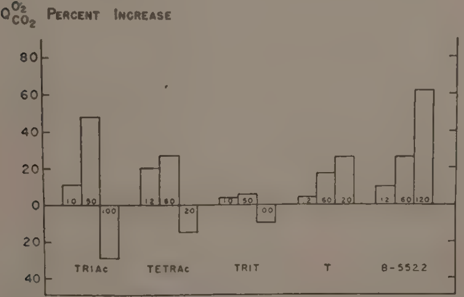

FIG. 1. Aerobic glycolysis of Ehrlich mouse ascites tumor cells as affected by thyroxine, Trit and related materials at various concentrations, shown as μg/ml.

If Trit is, as has been suggested, the "active" form of the thyroid hormone produced from thyroxine, it would seem unlikely that it is transformed into Triac prior to exerting its final actions. An alternate interpretation of these interconversions might be that any compound with the proper configuration is potentially active in its own right and that metabolism of thyroxine may yield a series of compounds, including Trit, Tetrac, Triac, and other derivatives.

Summary. Tetraiodothyroacetic acid (Tetrac) and triiodothyroacetic acid (Triac), in-

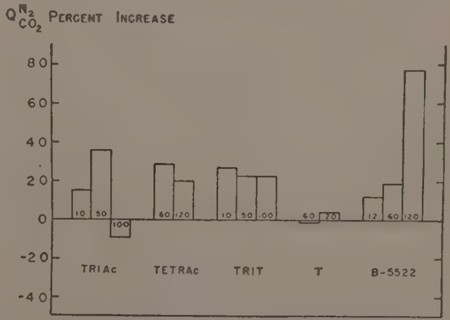

FIG. 2. Anaerobic glycolysis of Ehrlich mouse ascites tumor cells as affected by thyroxine, Trit and related materials at various concentrations, shown as μg/ml.

jected into thyroidectomized rats over a period of several days, have the same order of activity as an equimolar dose of L-thyroxine, as judged from the oxidative metabolism of excised tissues. Triac and Tetrac do not appear to produce an immediate metabolism-stimulating effect, either injected into animals or incubated *in vitro* with tissue slices. At levels which depress oxygen consumption of Ehrlich mouse ascites tumor cells, Triac and Tetrac enhance aerobic and anaerobic glycolyses. However, this effect is not considered specific to the acetate analogs, since thyroxine, triiodothyronine, and a physiologically inactive thyroxine isomer all are capable of producing similar changes.

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Radiation-Induced Alteration of Fibrinogen Clotting Rate and Clot Lability.* (22362)

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Studies of the effect of radiation on the biological activity of proteins have been confined almost exclusively to enzymes. In the case of the latter, an important body of information leading to a better understanding of radiation effects has been obtained(1). Investigations of proteins exposed to ionizing radiation have yielded a wealth of physicochemical data which must, however, be supplemented by examination of the radiation-induced modifications of their biological functions. In the light of the extensive work that has already been done on purified fibrinogen, it was decided to investigate the effects of x-radiation of fibrinogen on its subsequent clotting by thrombin. Fibrinogen has already been studied physicochemically after irradiation in the dry state(2) and in concentrated solution(3). In these studies, the doses of radiation employed were of such high magnitude as to have little practical significance from the point of view of mammalian radiation damage. The present study is concerned with the influence of low doses of radiation.

Materials and methods. Purified bovine fibrinogen (lot No. 55199) was generously supplied by Dr. E. C. Loomis of Parke, Davis and Co., and was shown to contain 90-93% clottable protein. Solutions of this fibrinogen were prepared in 0.15 M NaCl buffered with

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* This work was performed under U. S. Atomic Energy Commission contract AT(30-1) 1554, administered by L. V. Heilbrunn.

imidazole at pH 7.4 (final buffer concentration 0.015 M). Unless otherwise stated, the fibrinogen concentration in all experiments was 3 mg/ml. Single x-ray doses were given to freshly prepared fibrinogen solutions in Lusteroid tubes. The author wishes to express his gratitude to Messrs. J. L. Weatherwax and L. Stanton of the American Oncologic Hospital for kindly making available to him their x-ray facilities. The conditions of irradiation were 140 kilovolts, 5 milliamperes, 3 mm half-value layer filtration, and 20 cm target distance. The output of the machine was 150 r per minute. The clotting time, *i.e.*, the time required to attain firm-gel end-points, of irradiated and control solutions of fibrinogen, was determined in triplicate upon the addition of 0.05 ml of saline solution containing, unless otherwise stated, 0.063 unit of bovine thrombin (Topical, Parke, Davis). The clotting reaction took place in 12 x 75 mm Pyrex tubes contained in a water bath at 37.5°C. After allowing the fibrin clots formed in the above manner to remain in the water bath for at least one hour, an equal volume of 10 M urea was added. Each tube was shaken 3 times in order to free the clot from the bottom of the tube. The time for complete dissolution of the clot was then determined. In the experiments with sulfhydryl reagents the latter were added to the fibrinogen solutions prior to irradiation.

Results. Roentgen irradiation increased the clotting time of fibrinogen. The data are given in Fig. 1 and 2 where the logarithm of clotting time is plotted against the radiation dose. In Fig. 1 the dose range is from 0 to 500 r in steps of 100 r; each line represents a separate experiment. In Fig. 2 the dose range is 0 to 100 r in steps of 25 r, and here the 3 lines represent 3 different amounts of thrombin added. In both figures a linear relationship was obtained between the logarithm of the clotting time and the x-ray dosage. Inspection shows that the linear relationship between 0 and 100 r in Fig. 1 is verified by the data in Fig. 2. Fig. 3 indicates that the difference in clotting time between unirradiated and irradiated fibrinogen (500 r) varies inversely with the thrombin concentration. When 0.5 unit of thrombin are added, the difference in clotting time between the unirradiated and the irradiated solution is negligible. Thus, the radiation-induced clotting delay can be prevented by adding an excess of thrombin. The results of irradiation with 500 r on the clotting time of various concentrations of fibrinogen (1 mg/ml to 5 mg/ml) are plotted in Fig. 4. The clotting delay due to radiation was found to be independent of the fibrinogen concentration.

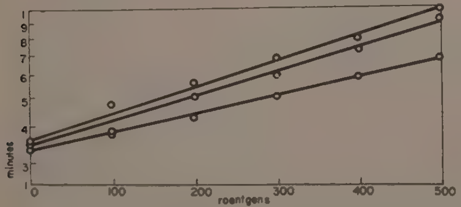

FIG. 1. Clotting time of fibrinogen as a function of radiation dose. Each curve represents a separate experiment.

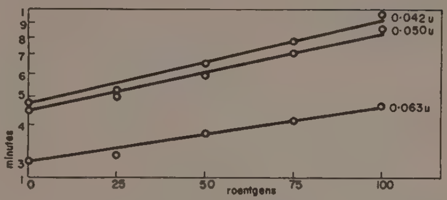

FIG. 2. Clotting time of fibrinogen as a function of radiation dose, using 0.063, 0.050, and 0.042 unit of thrombin, respectively.

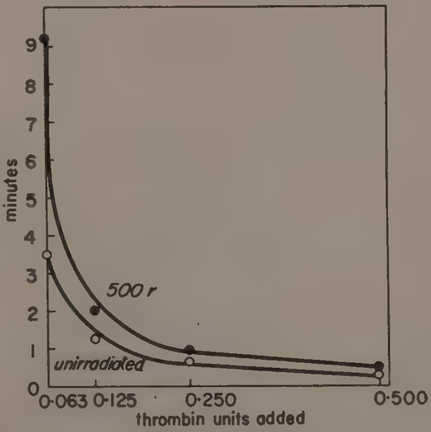

FIG. 3. Clotting time of fibrinogen as a function of thrombin concentration.

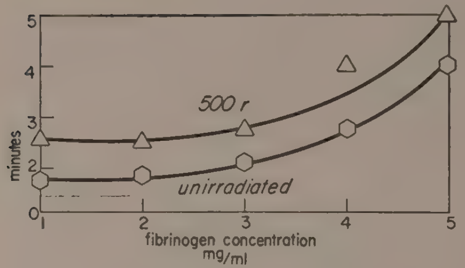

FIG. 4. Clotting time of fibrinogen as a function of fibrinogen concentration.

The extent to which the time of solution in 5 M urea of fibrin clots obtained from irradiated fibrinogen is shortened is indicated by Fig. 5. The plots of time of solution against x-ray dose, at 3 different concentrations of thrombin, reveal that when 0.25 unit of thrombin are added, there is no significant change. When the amount of added thrombin is decreased to 0.125 unit or to 0.063 unit, the time of solution decreases markedly as the x-ray dose increases. Thus, at thrombin concentrations where x-rays prolong the clotting time (Fig. 3), the time of solution of fibrin clots in urea is also decreased.

Addition to fibrinogen, prior to irradiation, of sulfhydryl compounds yielded the results summarized in Table I. 10^{-3} M cysteine or glutathione failed to abolish the radiation-induced clotting delay; they prolonged the clotting time in both the irradiated and unirradiated solutions. Thioglycollic acid (10^{-3} M), as well as higher concentrations of cysteine (10^{-2} M) completely inhibited the clotting process in both the irradiated and control solutions.

Discussion. The radiation-induced clotting delay in fibrinogen solutions may be attributed to a change in the fibrinogen molecule itself. That it is not due to a destruction of the latter will be reported in a subsequent communication, for irradiation failed to alter the amount of clottable protein. Indeed, even with the exposure of fibrinogen solutions to doses up to 130 kiloroentgens, Scherage and Nims (*op. cit.*) found practically no decrease in fibrin yield upon the addition of thrombin. They found that high doses of x-rays altered the fibrinogen to produce a polydisperse material with a higher sedimentation constant and increased viscosity. With the low doses of x-rays employed in the present study no change in viscosity was noted. Consequently, the radiation-induced alterations in the fibrinogen molecule exposed to low doses are of a much smaller magnitude than the effects observed by Scherage and Nims. Nevertheless, the effect of irradiation with low doses sufficed not only to delay the clotting time of fibrinogen, but also to produce structural alterations of the fibrin molecule as revealed by the decrease in time to dissolve fibrin clots in concentrated urea solution.

The progressive increase in clotting time due to x-radiation, leading finally to the establishment of an altered fibrin gel, could mean either that there has been a change in the initial polymerization of the activated fibrinogen, or that a modification, present in the fibrin strands, so changes the further polymerization as to give rise to an altered structural framework of the final clot. Both alternatives are probably at work to produce

TABLE I. Effect of Sulfhydryl Reagents on the Clotting Time of Unirradiated and Irradiated (500 r) Fibrinogen.

Additions	Conc. (M)	Control	Irrad.
None		4′ 30″	7′ 40″
Cysteine	8×10^{-3}	11′ 00″	18′ 10″
	4×10^{-2}	∝	∝
Glutathione (SH)	3×10^{-3}	8′ 15″	12′ 45″
Thioglycollic acid	2×10^{-3}	∝	∝

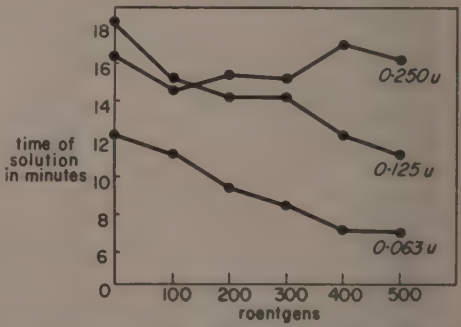

FIG. 5. Time of solution in 5 M urea of fibrin clots obtained from irradiated fibrinogen (500 r) clotted with 0.250, 0.125, and 0.063 unit of thrombin, respectively.

the results obtained. That at least the first possibility is involved can be inferred from the fact that, at high thrombin concentrations, the clotting delay due to radiation is no longer evident. At high thrombin concentrations there is an increase in the rate at which activated fibrinogen appears in solution(4). Apparently, at a high concentration of thrombin, the elaboration of normally activated fibrinogen is sufficiently rapid to overcome the radiation-induced reduction of polymerization. The increase in the difference in clotting time between the irradiated and the control solutions as the thrombin concentration is decreased is then a reflection of the decreased amounts of normally activated fibrinogen formed, the latter being replaced by increasing amounts of radiation-altered material. This modification of the fibrin strands produces an altered clot structure, since these strands comprise the network of a clot which is more highly accessible to the action of urea. At a relatively high thrombin concentration, this effect is also abolished, and this favors the interpretation that the primary modifications produced by radiation become manifest in the early stages of the clotting process.

The effect of low doses of radiation on fibrinogen is apparently not due to an oxidation of sulfhydryl groups, for as pointed out previously, sulfhydryl reagents failed to abolish the radiation-induced clotting delay. Nevertheless, these findings do not exclude the possibility that the polymerization phases of the clotting process are sulfhydryl dependent, since cysteine, glutathione, and thiourea protected radiation-sensitive sites in the fibrinogen molecule at doses ranging from 100 to 600 kiloroentgens(3). The persistence of clotting delay in the presence of similar concentrations of some of the same reagents indicates, however, that sulfhydryl reagents and x-rays act on separate sites in the fibrinogen molecule; both of these sites are essential for clotting to take place.

Summary. Purified bovine fibrinogen was exposed to single x-ray doses in the ranges of 25-100 r and 100-500 r. In each case the logarithm of the thrombin clotting time increased linearly with increasing dosage. The clotting delay due to irradiation was dependent on the thrombin concentration but independent of fibrinogen concentration. Addition, prior to irradiation, of sulfhydryl reagents failed to abolish the radiation-induced clotting defect. Irradiation of fibrinogen increased the rate of solution in concentrated urea of the fibrin clots obtained after addition of thrombin.

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Steroid Secretion by the Isolated Rat Adrenal.* (22363)

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(With technical assistance of Loma Coday.)

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Adrenal vein blood obtained from the rat has been demonstrated to contain several steroid hormones. Corticosterone is present in largest amount but hydrocortisone and aldosterone can also be detected(1,2). Bush found that the ratio of corticosterone to hydrocortisone secretion by the rat adrenal was approximately 20:1, whereas, in most other animals the secretion of hydrocortisone is greater than that of corticosterone(1). It has been demonstrated also that the rat adrenal gland secretes steroid hormones under *in vitro* conditions when stimulated by ACTH(3). Heard found that the rat adrenal incubated in the presence of ACTH secretes four steroids(4). On the basis of chromatographic mobility, ultraviolet light absorption, and staining reactions, these have been identified as corticosterone, cortisone, hydrocortisone, and an unknown substance.

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* This project was supported by grants from National Institute of Arthritis and Metabolic Disease, the National Vitamin Foundation, and the Williams-Waterman Fund.

The purpose of the present investigation was to determine the ratio of hydrocortisone and cortisone secretion to total steroid hormone production of the rat adrenal under *in vitro* conditions. To accomplish this, a method has been developed which permits quantitative determination of steroids containing an alpha-beta, unsaturated ketone structure in ring A and, also, steroids possessing a 17, 21 dihydroxy-20-ketone side chain. Thus, it is possible to determine the total steroid production of the isolated adrenal as well as the secretion of steroids such as hydrocortisone and cortisone. The results demonstrate that the secretion of hydrocortisone, cortisone, and other steroids possessing a dihydroxyketone side chain amounts to less than one-half of the total *in vitro* steroid production of the rat adrenal gland.

Procedure. Male Sprague-Dawley rats weighing 150-175 g were used in these studies. Animals were sacrificed by decapitation. The adrenals were quickly removed, dissected free of surrounding fat, weighed, and bisected. Each adrenal was placed in a beaker containing 2 ml of Krebs-Ringer-phosphate-glucose solution at pH 7.4. The glucose concentration in the medium was 200 mg%. One unit of ACTH was added to the contents of the beaker and the mixture incubated aerobically for 2 hours at 37°C in a Dubnoff metabolic incubator. On completion of incubation, the medium was decanted into a stoppered centrifuge tube leaving the adrenal slices in the beaker. The beaker was washed twice with one ml portions of distilled water and the rinsing water combined with the incubation medium in the centrifuge tube. The aqueous solution was extracted with 5 ml of ethylene dichloride and the mixture centrifuged at 1500 r.p.m. for 5 minutes. Following centrifugation, the aqueous phase was removed by aspiration and 4 ml of the ethylene dichloride extract was pipetted into a clean tube. The latter solution was taken to dryness under nitrogen at 50°C and the residue containing the steroids dissolved in 3 ml of methyl alcohol. Aliquots of the methyl alcohol solution were utilized for determination of the steroid concentration by a spectrophotometric technic and by the Porter-Silber method(5). Spectrophotometric measurement was accomplished by determining the absorption spectrum of the methanol solution from 220 to 260 mμ on a Beckman Model DU spectrophotometer. Steroids which possess an alpha-beta, unsaturated ketone structure in ring A demonstrate maximum absorption at 240 mμ. Steroid concentration was calculated from the absorption spectrum by the method of Allen (6). The Porter-Silber procedure measures only those steroids which have a 17,21 dihydroxy-20-ketone side chain such as cortisone, hydrocortisone, and Compound S.

Paper chromatographic separation of the steroid compounds secreted was achieved by combining the methyl alcohol solution obtained from 20 adrenal incubations. The toluene-propylene glycol chromatographic system of Zaffaroni was used(7). Twenty micrograms of compounds F, E and B were also placed on the chromatogram in order to compare the chromatographic mobility of the steroids secreted by the rat adrenal with that of known compounds. Steroids present on the chromatograms were detected by visualization under ultraviolet light and by staining with alkaline silver nitrate(7). Identification of the steroid secreted in largest quantity (steroid 4) was accomplished after elution of this substance from the chromatogram by use of the sulfuric acid chromogen technic(8).

Results. Recovery of steroids utilizing the method described was determined by adding known amounts of cortisone (free alcohol) to the incubation mixture. In these studies of

TABLE I. Recovery of Cortisone from Incubation Medium.

	Steroid recovery	
	Spectrophotometric method	Porter-Silber method
10.3 γ cortisone added	10.78 ± .19 γ* (104.7%) 11†	9.6 ± .11 γ* (93.2%) 12†

* Mean ± S.E. of mean.
† No. of determinations.

steroid recovery the adrenal slices were boiled to inactivate enzyme systems before being placed in the incubation medium. Table I demonstrates that recovery of cortisone was practically complete as measured by the spectrophotometric and Porter-Silber methods.

Table II compares the steroid secretion by isolated adrenals as measured by the spectrophotometric and phenylhydrazine - sulfuric acid methods. It is apparent that the spectrophotometric assay detects more than twice the quantity of steroid material determined by the Porter-Silber reaction.

TABLE II. Steroid Secretion by the Rat Adrenal Gland *In Vitro*; 19 Determinations in Each Series.

	Spectrophotometric method	Porter-Silber method
Actual steroid secretion/adrenal	11.4 ± 0.77 γ*	4.86 ± 0.24 γ
Steroid secretion per 100 mg adrenal wt	80.1 ± 5.28 γ	34.1 ± 4.58 γ

* Mean ± S.E. of mean.

The results of paper chromatographic separation of steroids secreted by rat adrenals are presented in Fig. 1. Four steroids are detected when the chromatogram is visualized under ultraviolet light and these are labelled in order of increasing mobility in the toluene-propylene glycol system. Steroid 1 has the same chromatographic mobility as does hydrocortisone. The mobility of steroid 2 is equal to that of cortisone. The third steroid band moves more rapidly than cortisone but slower than corticosterone. Steroid 4 moves at the same rate as does corticosterone in the toluene-propylene glycol system. Steroids 1, 2 and 4 reduce alkaline silver nitrate and it is, therefore, concluded that these steroids possess an alpha-ketol side chain.

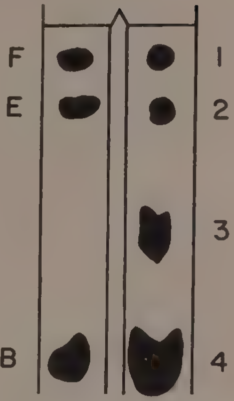

FIG. 1. Paper chromatographic separation of steroids secreted by isolated rat adrenal gland. Dark areas represent steroids which absorb ultraviolet light. Compounds F, E, and B were placed on left limb of chromatogram. The methanol solution containing the steroids secreted by 20 rat adrenals incubated with ACTH was placed on right limb of chromatogram.

Absorption spectra of the sulfuric acid chromogens of steroid 4 and that of corticosterone are presented in Fig. 2. It is apparent that the two spectral curves do not coincide. That of steroid 4 is, however, identical to the spectrum that has been reported for 11-epi-corticosterone (Δ 4-pregnene-11 α, 21 diol-3, 20 dione)(9).

Discussion. The investigations of Bush(1)

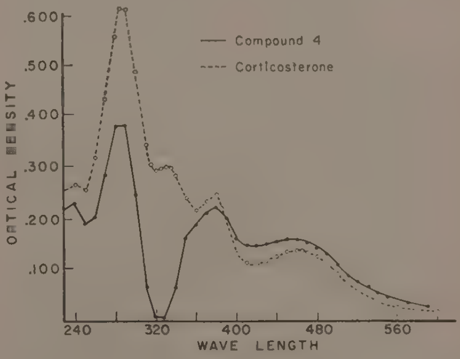

FIG. 2. Sulfuric acid chromogen absorption spectra of compound 4 and corticosterone. These steroids were eluted from the paper chromatogram, taken to dryness, and dissolved in concentrated sulfuric acid. Absorption maxima of compound 4 agree closely with those reported for 11-epi-corticosterone(9).

and Singer(2) have shown that adrenal vein blood obtained from the rat contains several steroid hormones including corticosterone, aldosterone, hydrocortisone, 11-dehydrocorticosterone, and 11-hydroxyandrostenedione. The steroid secreted in greatest quantity is corticosterone and the other hormones are produced in small amounts. Heard(4) has stated that the isolated rat adrenal cortex secretes both hydrocortisone and cortisone as well as corticosterone and an unknown substance. The results obtained in the studies reported herein also show that four steroids containing the alpha-beta, unsaturated ketone structure are secreted by the isolated rat adrenal. Quantitative measurement of steroid secretion demonstrates that almost one-half of the total adrenal hormones secreted under *in vitro* conditions consists of steroids which possess the 17, 21 dihydroxyketone side chain. The secretion of hydrocortisone, however, must account for only a small portion of the Porter-Silber reactive material since the area of the chromatogram containing this steroid was relatively small. If compound 2 is cortisone, as suggested by Heard, this would account for an additional quantity of steroid containing the dihydroxyketone side chain. The secretion of cortisone, however, was also small as judged by the area of the chromatogram containing this steroid. It is, therefore, possible that at least a portion of the steroid material present in area 3 contains a 17, 21 dihydroxy-20-ketone side chain. Heard(4) has obtained evidence which indicates that there are at least 2 steroids present in area 3 and believes that one of these is Compound S, a steroid which has a dihydroxyketone side chain and would, therefore, be measured by the Porter-Silber technic.

It has been demonstrated that corticosterone is the major hormone secreted by the rat adrenal *in vivo*(1). The steroid secreted in greatest quantity in these experiments possessed a chromatographic mobility equal to that of corticosterone. In addition, this compound, like corticosterone, contains an alpha-beta, unsaturated ketone in ring A and has an alpha-ketol side chain. However, the product formed by addition of sulfuric acid to this substance demonstrated an absorption spectrum different from corticosterone but identical to that reported for 11-epi-corticosterone. It is, therefore, concluded that under *in vitro* conditions the chief hormone secreted by the rat adrenal is 11-epi-corticosterone.

The explanation of the finding that the isolated rat adrenal secretes 11-epi-corticosterone, while under *in vivo* conditions corticosterone is produced, is not apparent. Previous investigations, however, have shown that there is a discrepancy between *in vivo* and *in vitro* enzymatic reactions concerned with steroid hormone metabolism. Dorfman (10) has demonstrated that if C_{21}-Δ^4-3 ketosteroids are incubated with liver or adrenal tissue, the reduction products formed are in the 5-alpha series. When these compounds are administered to the living animal, the reduction products obtained have the 5-beta configuration. Since in these investigations it has been demonstrated that an epimer of corticosterone is secreted by the isolated rat adrenal cortex, it is suggested that some of the other hormones produced *in vitro* may also be stereoisomers of the compounds secreted *in vivo*.

Summary. (1) A method to determine the *in vitro* steroid secretion by the rat adrenal is described. This procedure measures total steroid hormone production as well as secretion of steroids which have a 17, 21 dihydroxy-20-ketone side chain. (2) It has been demonstrated that the *in vitro* secretion of adrenal hormones which possess a dihydroxyketone side chain amounts to almost one-half of the total steroid secretion. (3) The isolated rat adrenal secretes four steroid hormones in response to ACTH. The compound secreted in largest amount has been shown to be 11-epi-corticosterone.

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Iodine Metabolism in a Region of Endemic Goiter. (22364)

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The great avidity for iodine exhibited by the thyroid gland of subjects living in certain regions where endemic goiter is prevalent has been demonstrated by Stanbury *et al.*(1,2) and by Roche *et al.*(3). It appeared to us of interest to carry out further measurements on different aspects of iodine metabolism in the regions previously studied by us. Results of thyroid and renal radioiodine clearance, conversion ratios, protein-bound I^{131}, stable iodine urinary excretion, rate of hormonal iodine formation and salivary radioactivity are reported in the present article.

Material and methods. All subjects were apparently healthy and euthyroid adults. One group (cases 1 to 14) came from the region of Bailadores, where previous studies had been carried out(3), and the other group (cases 15 to 21) from the region of Tabay, near the city of Mérida. Both regions lie within the Venezuelan Andes, at an altitude above 4,500 feet. For the study of thyroid I^{131} uptake, subjects were chosen whose thyroid was either barely palpable, or palpable but not readily visible. Radioiodine was supplied by Abbott Laboratories at Oak Ridge. External thyroid measurements were carried out with a Nuclear (Model DS-1) scintillation counter, placed 40 cm away from the thyroid cartilage. The resulting activity was multiplied by 0.85(1), and the activity measured 40 cm from the thigh was subtracted from the resulting value. The result was expressed as % of the administered dose, as measured 40 cm away from a 250 cc beaker (when the dose was given orally) or in a 20 cc syringe (when the dose was given intravenously). The beaker was thoroughly washed and the washings given to the patients. The syringe was counted before and after intravenous administration of the dose, and the second count was subtracted from the first. Measurements of plasma, saliva and urine radioactivity were performed in a well-type scintillation counter (Nuclear, Model 3037 B) and compared with the activity of suitably prepared standards.

Measurements of thyroid and renal clearances were carried out as follows: 50 μc of I^{131} in 10 cc of saline solution were injected intravenously. The thyroid was counted at 2 and 29 minutes during 2 consecutive minutes. The results, properly corrected, were taken to represent neck activity at 3 and 30 minutes respectively, and were expressed in terms of % of the administered dose. Blood was obtained at 5, 10 and 30 minutes and radioactivity of 3 cc of plasma was counted, compared with a standard and expressed in terms of % of the administered dose per liter of plasma. The patient was asked to urinate at 35 minutes and urine radioactivity was measured and compared with the standard. Thyroid clearance was calculated from a formula given by Kretchmar(4), modified from the method of Berson *et al.*(5):

$$T_c = \frac{N_{30} - \left[\dfrac{\dfrac{P_{30}}{P_3} \times N_3}{0.88}\right]}{\mu}$$

Where T_c = Thyroid clearance, N_{30} = Neck activity at 30 min., N_3 = Neck activity at 3 min., P_{30} = Plasma activity at 30 min., P_3 = Plasma activity at 3 min., calculated from curve constructed with the 5, 10 and 30 minute values and μ = Average plasma level, as measured planimetrically from the plasma radioactivity curve. The factor 0.88 was taken from Oddie(6). Renal clearance was calculated by dividing average plasma dose by urine concentration. Conversion ratio was established by counting radioactivity in 3 cc of plasma, precipitating with 6 cc 10% trichloracetic acid, washing twice with the same substance, and redissolving the precipitate in 3 cc 1 N NaOH. Saliva was obtained without stimulation, and the radioactivity was counted in the deep well scintillator. Urinary I^{127} concentration was determined chemically (7). The urines were collected in the usual way, their volume was measured and an aliquot was alkalinised, placed in glass-stoppered bottles, kept in the ice box and taken directly by plane within three weeks to the Boston Medical Laboratory where the determinations were performed. Rate of hormonal iodine secretion was calculated from the formula given by Stanbury *et al.*(2).

TABLE II. Conversion Ratios and Protein-Bound I^{131} in Subjects from Endemic Goiter Region.

Patient	Sex	Type of goiter*	Conversion ratio (%)	24 hr protein-bound I^{131} (% dose/L of plasma)
8	♂	D 1	96	.017
9	♂	D 2	97	.021
10	♂	N 2	100	.019
11	♂	D 2	42	.011
12	♀	N 2	52	.020
13	♂	D 2	90	.012
15	♂	D 2	51	.048
17	♀	N 3	75	.112
18	♀	N 3	90	.084
19	♀	N 3	64	.045
20	♀	D 1	87	.522
21	♂	N 3	87	.056
		Avg	78	.081
		Range	42–100	.011–.522

* N = Nodular; D = Diffuse. 1 = Barely palpable; 2 = Definitely enlarged, but not yet visible; 3 = Enlarged and visible.

Results. In Table I are shown results of thyroid uptake and clearance, renal 24 hr excretion and clearance of I^{131}, 24 hour I^{127} urinary excretion and daily rate of hormonal iodine secretion. All the patients in this table came from the region of Bailadores.

In Table II are listed the results of conversion ratios and 24 hour protein-bound I^{131}. In Table III we may see the results of sali-

TABLE I. Studies in Iodine Metabolism in Subjects from a Region of Endemic Goiter.

Patient No.	Type of goiter	Sex	24 hr thyroid I^{131} uptake (% of dose)	24 hr urinary I^{131} (% of dose)	Thyroid I^{131} clearance (ml/m)	Renal I^{131} clearance (ml/m)	Urinary I^{127} excretion (μg/d)	Rate of hormonal iodine secretion (μg/d)
1	No	♂	49.0	31.8	118.5	58.0	21.8	20.9
2	No	♂	54.8	25.9	124.4	43.7	19.8	24.0
3	N 2	♂	56.2	—	—	—	—	—
4	D 2	♂	52.0	33.2	82.6	35.7	9.6	10.4
5	D 1	♂	54.6	39.7	135.4	35.8	26.7	32.1
6	D 2	♂	55.3	15.3	130.5	42.7	19.8	24.5
7	D 2	♀	—	—	168.2	21.8	—	—
8	D 1	♂	43.2	13.2	86.2	51.5	21.1	16.0
9	D 3	♂	59.0	18.3	131.0	8.0	19.2	27.6
10	N 2	♂	53.0	22.7	110.3	18.8	20.0	22.6
11	D 2	♂	41.5	52.2	87.2	26.8	40.0	28.3
12	N 2	♀	36.1	79.5	38.5	35.3	35.4	20.0
13	D 2	♂	57.6	30.1	125.5	72.0	27.3	37.1
14	D 1	♂	43.1	12.5	70.7	32.0	9.0	6.8
Avg			50.4	31.2	108.4	37.1	22.5	22.5
Range			36.1–59.0	12.5–79.5	38.5–168.2	8.0–72.0	9.0–40.0	6.8–37.1

TABLE III. Saliva Radioactivity 24 Hr after Administration of 50 μc of I^{131}.

Patient No.	Saliva radioactivity (c/m/3 cc)
1	1262
2	713
3	1410
4	730
5	657
6	792
8	1412
9	727
10	961
11	1246
12	4670
13	654
14	2539
15	1604
16	640
17	2930
18	256
19	690
20	2819
21	91
Avg	1341
Range	91–4670

vary radioactivity measurements.

Discussion. The I^{131} studies are in accord with previous reports(2,3) and may be explained by a high iodine extracting efficiency of the thyroid. Thyroid clearance in these cases is generally well above the normal limits of 8-38 ml/m (mean 16) given by Myant *et al.*(8), of 26.3 ± 5.2 (standard error) given by Oddie *et al.*(6), and of 12.0-68.0 (mean 33.0 ml/m) given by Berson *et al.* (5). Although the 24 hour thyroid I^{131} uptake is not greatly elevated, and in some of the cases is even normal, a study of the thyroid clearance shows clearly that thyroid avidity for iodine is markedly increased in this region. In individuals with larger goiters, one would expect this avidity to be even higher. The only case who shows nearly normal values (case No. 12) was a woman who had lived for 15 years in Caracas, the capital, where thyroid uptake has been shown to be normal(9), and had lived in Bailadores for the last 3 years. Her diet was plentiful as compared to that of the other subjects in this study whose diet was almost exclusively of local vegetal origin(11).

Renal I^{131} excretion is somewhat reduced, but clearance is normal, showing that in these regions individuals do not develop the ability to conserve iodine through renal mechanisms. Normal renal iodine clearance is given by various authors as 11-44 ml/m, with a mean of 31 ml/m(8), 31.0-76.0 ml/m with mean 53.9(5) and 33.3 ± 3.1 (standard error)(10). Stanbury *et al.*, in Mendoza, in 3 subjects, found renal clearance values of 25, 29 and 44 ml/m respectively, all within normal limits also(2).

In cases 6, 8 and 14, the very low I^{131} excretion is suspicious, since total recovery of I^{131} was low; loss of urine is probable. Individual urine volumes in these cases were 1,100, 1,320 and 900 cc.

The 24 hour stable iodine excretion is quite low, confirming further the probability that the finding of increased thyroid avidity for iodine in this region is conditioned by iodine deficiency in food and water. Compensation for this iodine deficiency appears to be adequate in the cases studied, as evidenced by the clinical status and by the normal, although low, daily hormonal iodine secretion. A similarly low I^{127} excretion (17 μg/day average) had been found previously by De Venanzi *et al.* in individuals from a region nearby (13).

Saliva radioactivity has not been previously reported in a region of endemic goiter. In our cases, it was generally low, and corresponded approximately to the values one would expect in hyperthyroid patients with similar thyroid uptakes(12).

Conversion ratios are unusually high, and there seems to be an increased quantity of protein-bound I^{131} at 24 hours in the plasma. Here again, the findings resemble those of hyperthyroidism and are compatible with the idea that in the endemic goiter found in this region, as in the Mendoza goiter, there is increased extraction of iodine by the thyroid, a faster rate of iodine turnover, with an adequate quantity of hormonal iodine being synthesized.

Summary. Studies carried out in the Venezuelan Andes show that in this region there is an increase in thyroid clearance of iodine, thyroid uptake, conversion ratios and 24 hour protein-bound I^{131}. Renal clearance of I^{131} is normal and urinary and salivary I^{131} low. Urinary I^{127} is low. It is concluded that in this region there exists a state of iodine deficiency, leading to compensatory thyroid avidity for iodine. There is no compensatory renal conservation of iodine.

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